Proteasome activator PA28γ regulates p53 by enhancing its MDM2-mediated degradation

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Downregulation of p53 by MDM2-mediated proteasomal degradation makes cells resistant to apoptosis. The MDM2–p53 interaction is well characterized, but the mechanisms that regulate the interaction are not well understood. Here, we show that PA28γ, a proteasome activator that inhibits apoptosis and promotes cell cycle progression through unknown mechanisms, exerts an effect as a cofactor in the MDM2–p53 interaction. The polymer form of PA28γ interacts with both MDM2 and p53 proteins and facilitates their physical interaction. This promotes ubiquitination- and MDM2-dependent proteasomal degradation of p53, limiting its accumulation and resulting in inhibited apoptosis after DNA damage. Elimination of endogenous PA28γ in human cancer cells abrogates MDM2-mediated p53 degradation, increases the activity of p53, and enhances apoptosis. These findings reveal the mechanism by which PA28γ affects apoptosis and proliferation. Manipulation of the level of PA28γ, an approach that would regulate the cellular content of p53, may improve the efficacy of current cancer therapies.

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Introduction

Tumor suppressor p53 is involved in controlling cell growth and differentiation and in the maintenance of genomic integrity, with high levels of p53 activity inducing apoptosis, cell cycle arrest, or senescence (Lane, 1992). The regulation of p53 protein expression and activity in cells is of particular interest in the field of cancer research, both for the development of more effective therapeutic strategies and for ongoing efforts to gain a better understanding of tumorigenesis. The level of p53 protein is elevated upon exposure of cells to stress, such as DNA damage, hypoxia, nutrient deprivation, or oncogene activation (Levine, 1997). In unstressed cells, the level of p53 (hence its activity) is largely controlled by MDM2, an oncoprotein containing an ubiquitin E3 ligase (Haupt et al., 1997; Brooks and Gu, 2006). MDM2 promotes the polyubiquitination of p53, which is an essential step in its degradation (Haupt et al., 1997; Brooks and Gu, 2006).

Although it is known that the MDM2–p53 interaction is controlled by an autoregulatory loop (Haupt et al., 1997; Brooks and Gu, 2003, 2006; Zhang et al., 2005) and that the MDM2-mediated degradation of p53 is dependent upon the cellular level of MDM2 (Li et al., 2003), the mechanisms regulating this interaction appear to be complex and are poorly understood. It was suggested that a cofactor may be required for MDM2-mediated p53 degradation because low levels of MDM2 fail to catalyse polyubiquitination of p53 (Li et al., 2003).

PA28γ (REGγ, PSME3, Ki antigen) belongs to a family of activators of the 20S proteasome (Rechsteiner and Hill, 2005). Unlike its family members, PA28α and PA28β, PA28γ is localized within the nucleus and forms a homohexamer (rather than a heterocomplex). PA28γ has been implicated in the regulation of cell cycle progression and apoptosis, and embryonic fibroblasts of PA28γ nullizygous mice demonstrate spontaneous apoptosis and G1 arrest (Rechsteiner and Hill, 2005). These mice also have a reduced adult body mass and show growth retardation (Rechsteiner and Hill, 2005). The mechanism by which PA28γ exerts these effects has not been elucidated. These findings prompted us to explore the possibility that PA28γ may regulate p53. Here, we report that PA28γ has an integral function in the degradation of p53, and that it does so by acting as an essential cofactor that promotes the binding of MDM2 and p53.

Results

PA28γ negatively regulates p53
Overexpression of PA28γ, but not PA28β, reduced the level of p53 protein and its transactivational activity (measured by p21Waf1 protein) in LNCaP, MCF-7, and U2OS cells (p53 wild type), but not in PC3 cells (p53 null) (Figure 1A). Knockdown of endogenous PA28γ using a small interfering RNA (siRNA) pool (Dharmacon) increased the levels of endogenous p53 and p21Waf1 in LNCaP, HCT116, A549, MCF-7, and MCF10A (a normal breast epithelial line) cells, but not PC3 cells (Figures 1B–D and Supplementary Figure S1). Although PA28γ levels were decreased in all eight cell lines treated with PA28γ siRNA (Figures 1B and C and Supplementary Figures S1 and S2): levels of the homologue PA28β showed little or no change, indicating that the off-target effects of the siRNA pool were minimal (Figure 1B and Supplementary Figure S1).

Confirming that induction of p21Waf1 after PA28γ knockdown is p53 dependent, when MCF10A cells were transfected...
with an siRNA pool targeting PA28γ, p53, or both, the induction of p21Waf1 was largely inhibited when p53 was knocked down (Figure 1C). Furthermore, an increase in p21Waf1 mRNA after PA28γ knockdown by siRNA was observed in HCT116 cells, but not in p53−/− HCT116 cells (Figure 1D). At the post-translational level, the extent of the induction of p21Waf1 in p53−/− HCT116 cells was much less than in HCT116 cells (Figure 1D). These results suggest that the induction of p21Waf1 after PA28γ knockdown occurs largely through p53. However, modulation of p21Waf1 directly by PA28γ at the post-translational level is also possible.

Two individual PA28γ siRNA duplexes and one control siRNA duplex were randomly selected from the previous siRNA pools to confirm that PA28γ negatively regulates p53. Each of the PA28γ siRNA duplexes was capable of down-regulating endogenous PA28γ, resulting in dose-dependent accumulation of p53 in HCT116 cells (Figure 1E, left panel). In contrast, randomly selected PA28β siRNA duplexes from the PA28β siRNA pool (Dharmacon) did not affect the level of p53, although endogenous PA28β was knocked down (Figure 1E, right panel). Additionally, p53 was elevated in PA28γ−/− mouse embryonic fibroblasts (MEFs) (with low passage numbers) compared with wild-type MEF (Figure 1F). This elevation in p53 was decreased by overexpressed PA28γ (Figure 1G).

Several p53 targets (including p21Waf1, Puma, and 14-3-3σ), and p53 itself, were downregulated by ectopic PA28γ in wild-type MEF cells (Figure 1H). In HCT116 cells, knockdown of PA28γ led to induction of p53 at the protein level and increases in p21Waf1, Puma, and 14-3-3σ at both the mRNA and protein levels (Figure 1I). Similar results were obtained in MCF-7 cells (Supplementary Figure S3). Overexpression of PA28γ, but not PA28β, in MCF-7 cells co-transfected with a luciferase reporter under the control of the p21Waf1 promoter, inhibited p53-dependent transcriptional activity (Figure 1J, left panel). Co-transfection with p53 reversed the PA28γ-mediated inhibition of p53-dependent transcriptional activity (Figure 1J, right panel).

**PA28γ promotes ubiquitination-dependent proteasomal degradation of p53**

We hypothesized that PA28γ downregulates p53 by promoting its degradation. Confirming this hypothesis, overexpression of PA28γ increased degradation of endogenous p53 protein in LNCaP cells (Figure 2A), and increased the amount of proteasome-bound p53 in p21Waf1-overexpressing U2OS cells (Figure 2B). In MCF-7 cells, overexpression of PA28γ enhanced the ubiquitination of p53 (Figure 2C).

The stability of p53 in ts20b cells, which express a temperature-sensitive E1 ubiquitin-activating enzyme, was examined to determine whether the promotion of p53 degradation by PA28γ is ubiquitin-dependent. PA28γ lost its capacity to promote p53 degradation when the ts20b cells were cultured at 39°C. However, its promotion of ubiquitin-independent proteasomal degradation of p21Waf1 was retained (Figure 2D).

To exclude the possibility that PA28γ may have a post-ubiquitination effect in promoting degradation of ubiquitinated p53 through the proteasome, ubiquitinated p53 was incubated with either 20S or 26S proteasome in the presence of PA28γ. The 20S proteasome alone did not degrade ubiquitinated p53, and PA28γ did not activate the 20S proteasome to degrade ubiquitinated p53 (Figure 2E, upper panel). Although the 26S proteasome degraded ubiquitinated p53, PA28γ did not enhance this activity (Figure 2E, lower panel). In contrast, PA28γ enhanced the degradation of p21Waf1 by the 20S proteasome (Figure 2F).

**PA28γ is a cofactor for MDM2-mediated p53 degradation**

The PA28γ protein does not have a motif characteristic of an ubiquitin E3 ligase, suggesting that PA28γ may promote the ubiquitinating activity of another molecule. To test this hypothesis, we examined whether PA28γ promotes MDM2-mediated p53 ubiquitination and degradation. Co-transfection of low amounts of MDM2 and PA28γ into MCF-7 cells enhanced the downregulation of p53 (Figure 3A). Although low amounts of PA28γ or MDM2 transfected alone into LNCaP cells (Figure 3B) or A549 cells (Figure 3C) minimally induced p53 ubiquitination, co-transfection with both PA28γ and MDM2 enhanced its ubiquitination (Figures 3B and C). The possibility that the enhanced ubiquitination was a result of changes in the pool of cellular-free ubiquitin, E1 or E2 was excluded using purified recombinant proteins in in vitro ubiquitination assays. Incubation of a lower amount of MDM2 protein with purified p53 protein, along with purified E1, E2 and ubiquitin in vitro, resulted in minimal ubiquitination of the p53 protein (Figure 3D). When recombinant PA28γ was included in the reaction, the ubiquitination of p53 was increased (Figure 3D).

 Exposure to a high concentration of Nutlin-3 (10μM), a known inhibitor of the MDM2–p53 interaction, 12 hours after transfection of MCF-7 cells with PA28γ reversed the effects of PA28γ on p53 (Figure 3E). Exposure of LNCaP cells to an anti-MDM2 antisense oligonucleotide (AS) (Zhang et al, 2005), but not its mismatch control (ASM), prevented the induction of p53 degradation by PA28γ (Figure 3F). Co-transfection of U2OS cells with MDM2 ASM or AS and various amounts of PA28γ further indicated that the PA28γ-mediated reduction in the p53 level is dependent on the presence of MDM2 (Figure 3G). When PA28γ was knocked down in A549 cells by siRNA prior to transfection with MDM2, the down-regulatory effect of MDM2 on p53 was not observed (Figure 3H).

The kinetics of the changes in expression and transactivational activity of p53 after γ-irradiation were compared in A549 cells overexpressing either MDM2 or PA28γ (Figure 3I). Both MDM2 and PA28γ limited the accumulation of p53 and the elevation of p53 activity (manifested by the level of p21Waf1) with similar kinetics after DNA damage (Figure 3J), indicating that they work via the same pathway. When PA28γ was pre-knocked down by siRNA, neither a low concentration of Nutlin-3 (3μM) (Figure 3J) nor overexpressed p14ARF (Figure 3K) led to modulation of the level of p53. This is likely due to the fact that the MDM2–p53 interaction (the target of both Nutlin-3 and p14ARF) is disrupted by the absence of PA28γ. Another cofactor for the MDM2–p53 interaction, Daxx, also lost its capacity to downregulate p53 in the absence of PA28γ (Figure 3L). In contrast, YY1 retained its capacity for downregulating p53 (Figure 3M), indicating that the mechanism by which YY1 enhances the MDM2–p53 interaction is different from that of PA28γ.
PA28γ binds to MDM2 and p53, and promotes the MDM2–p53 interaction

The stability of MDM2 did not change in PC3 cells transfected with either PA28γ or the control vector (Supplementary Figure S4), suggesting that it is unlikely that PA28γ modulates the level of p53 by inhibiting the capacity of MDM2 to catalyse self-ubiquitination. As PC3 cells are p53 null, changes in MDM2 are not a result of alterations in p53 induced by PA28γ.

Direct physical interactions between endogenous PA28γ and p53 proteins, as well as between endogenous PA28γ and MDM2 proteins, were observed in MCF-7 cells (Figure 4A). Confirmation of these direct interactions was obtained by use of pull-down assays (Figure 4B), and immunoprecipitation
with overexpressed PA28γ–GFP and p53-HA (haemagglutinin) (Figure 4C, upper panel) and PA28γ–GFP and MDMD-T7 (Figure 4C, lower panel) in COS7 cells.

To determine if PA28γ promotes the MDMD–p53 interaction, lysates from U2OS cells overexpressing different amounts of PA28γ were immunoprecipitated with p53 antibody, and it was observed that the MDMD level was higher in the p53 immunoprecipitates from cells that were transfected with PA28γ (Figure 4D, left panel). Moreover, the amount of MDMD in p53 immunoprecipitates from A549 cells treated with the PA28γ siRNA pool was lower than that from cells treated with the control siRNA pool (Figure 4D, right panel). The amount of p53 bound to GST-MDM2 was also enhanced by the addition of PA28γ in an in vitro pull-down assay (Figure 4E). Finally, the amount of MDMD bound by p53 in PA28γ−/− MEFs was higher than in wild-type MEF cells (Figure 4F).

To determine which form of PA28γ (monomer/polymer) mediates the MDMD–p53 interaction, gel filtration chromatography was performed. The elution profile of the lysates from COS7 cells transfected with GFP–PA28γ, p53-HA, and MDMD-T7 showed a peak that was absent in the profile from the cells transfected with GFP, p53-HA, and MDMD-T7 (Figure 4G, left panel). The molecular weight of this peak was estimated to be 560 kDa, corresponding to a complex of heptameric GFP–PA28γ–MDMD–p53 (the molecular weight of GFP–PA28γ is 60 kDa) (Figure 4G, left panel). The eluted fractions were resolved on 10% SDS–PAGE and immunoblotted using T7, HA, and GFP antibodies (Figure 4G, right panel).

In GFP–PA28γ-transfected cells, GFP–PA28γ, MDMD–T7, and p53-HA appeared simultaneously in the fractions with molecular weights from about 440 kDa to more than 669 kDa (Figure 4G, right panel). Moreover, GFP–PA28γ barely appeared in the fractions with molecular weights less than 232 kDa, suggesting that GFP–PA28γ primarily exists in the polymer form in cells. In GFP-transfected cells, p53-HA and MDMD–T7 appeared simultaneously only in the fractions with lower molecular weight, about 400–440 kDa (Figure 4G, right panel). To assess the capacity of GFP–PA28γ to form polymers, an in vitro approach employing glutaraldehyde (to covalently crosslink any polymers that form) was performed (Supplementary Figure S5). GFP–PA28γ and three mutants, P245Y, G150S, and N151Y (incapable of activating the proteasome), were all capable of forming polymers (Supplementary Figure S5).

**PA28γ specifically downregulates p53**

To confirm that PA28γ does not regulate p53 directly through its proteasome-binding or -activating functions, we tested the activity of three mutants of PA28γ: P245Y (incapable of binding the 20S proteasome; Zhang et al, 1998b), and G150S and N151Y (unable to activate, but still able to bind the proteasome; Zhang et al, 1998b). Similar half-lives for endogenous p53 protein were found in cells that were transfected with PA28γ and cells transfected with any of the three mutants (Figure 5A). Notably, the homologue-specific ‘insert region’ of PA28γ (amino acids 71–103) that differentiates it from other PA28 family members (Zhang et al, 1998b) was demonstrated to be essential for the promotion of poly-ubiquitination and degradation of p53 (Figures 5A–C).

We have further defined the regions that are essential for the binding of PA28γ to MDMD and p53, both of which are within the insert region. A PA28γ mutant without amino acids 76–103 did not bind to MDMD, but PA28γ 66–161, PA28γ 86–255, and PA28γ 96–255 all bound MDMD (Figures 5D, F and Supplementary Figure S6A), indicating that amino acids 96–103 are essential for PA28γ binding to MDMD (Figures 5D, F and Supplementary Figure S6A). We also determined that amino acids 86–96 are required for binding p53 (Figures 5E, F and Supplementary Figure S6B).
PA28γ modulates MDM2–p53 interaction
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Figure 2 PA28γ promotes ubiquitination-dependent p53 proteasomal degradation. (A) PA28γ destabilizes p53 protein in cells. LNCaP cells were transfected with PA28γ (4 μg) or corresponding empty vector for 24 h, followed by exposure to cycloheximide (CHX) (10 μg/ml) for different times. Target proteins in whole cell lysates were detected by immunoblotting. The intensity of the p53 protein band was analysed by densitometry (Bio-Rad Gel Doc). The relative densitometry data at each time point are expressed as a percentage of the density at time 0, after normalization to the corresponding β-actin level. The average relative densitometry data of three independent experiments are shown in the right panel. *P<0.05. (B) PA28γ induces proteasome binding of p53. U2OS cells with PA28γ (0 or 4 μg) were evaluated for proteasome-bound p53 by immunoprecipitation with an antibody to the proteasome α/β subunits followed by detection of p53 by immunoblotting with p53 antibody DO-1. Proteasomal ATPase subunit Rpt1/S7 was included to verify equal protein loading. (C) p53 ubiquitination is enhanced in cells with PA28γ overexpression. HA-p53 (3 μg) and PA28γ (3 μg) were overexpressed in MCF-7 cells followed by exposure to MG132. Ubiquitinated p53 was detected by direct immunoblotting with the DO1 antibody. (D) The promotion of p53 degradation by PA28γ depends on the integrity of the ubiquitin–proteasome pathway. ts20b cells were incubated at 32 or 39°C for 12 h before being transfected with plasmids to overexpress PA28γ (3.5 μg) or control vector. The stability of p53 and p21Waf1 proteins was determined 24 h later as described in (A). (E) PA28γ does not promote proteasomal degradation of ubiquitinated p53. Ubiquitinated p53 was incubated with 20S or 26S proteasome in the presence or absence of PA28γ at 37°C for 5 h. The reaction was terminated by boiling with SDS sample buffer for 5 min. Ubiquitinated p53 was detected by immunoblotting. (F) PA28γ promotes p21Waf1 degradation by the 20S proteasome. p21Waf1 was incubated with 20S proteasome in the presence or absence of PA28γ at 37°C for 45 min. The reaction was terminated by boiling with SDS sample buffer for 5 min. p21Waf1 was detected by immunoblotting.

Figure 3 PA28γ functions as a critical cofactor for MDM2-mediated p53 degradation. (A) MDM2 and PA28γ synergistically decrease p53 protein. MCF-7 cells were transfected with low amounts of MDM2 (0, 1, or 1.5 μg) in the presence or absence of PA28γ (0 or 1.5 μg) for 24 h. Endogenous p53 protein expression was whole cell lysates evaluated by immunoblotting. (B, C) MDM2 and PA28γ synergistically induce p53 ubiquitination in cells. (B) Different combinations of MDM2 (0 or 2 μg), Ub-HA (0 or 2 μg), and/or PA28γ (0 or 2 μg) were expressed in LNCaP cells. Ubiquitinated p53 was immunoprecipitated using p53 antibody DO-1. and the precipitates were immunoblotted using antibodies against HA and ubiquitin. *Ubiquitinated p53. (C) AS49 cells were transfected with MDM2, Ub-HA, and PA28γ as described in (B). 24 h later, cells were incubated with MG132 (15 μM) for 4 h. Cell lysates were immunoprecipitated by p53 antibody FL393, and the precipitates were immunoblotted with an antibody against the polyubiquitin chain, FK1. In vitro ubiquitination of p53 by MDM2 is enhanced by PA28γ. His-p53 (6 ng) was incubated with His-MDM2 (3 ng) and GST-PA28γ (30 ng) for 3 h in the presence of E1 (10 ng), E2 (40 ng), and ubiquitin (5 μg). Ubiquitinated p53 was detected by immunoblotting with the p53 antibody, DO-1. (E) The downregulation of p53 by PA28γ is reversed by Nutlin-3. (F) MCF-7 cells were transfected with the PA28γ plasmid (3.5 μg) or control vector (3.5 μg), and incubated for 24 h. At 12 h before the termination of the incubation, cells were exposed to 10 μM Nutlin-3 or the solvent, DMSO. Target proteins in whole cell lysates were detected by immunoblotting. (G) PA28γ loses the capacity to induce p53 degradation when MDM2 is knocked down by MDM2 antisense oligonucleotides. (F) LNCaP cells were co-transfected with PA28γ (4 μg) or control vector (4 μg) with an MDM2 antisense oligonucleotide (AS) (150 nM) or a mismatch control oligonucleotide (ASM) (150 nM). After 24 h, p53 stability was determined as described above. (G) U2OS cells were co-transfected with MDM2 AS (150 nM) or ASM (150 nM) with various amounts of PA28γ (0, 1, 2, 3, or 4 μg). The p53 protein level in cell lysates was determined by immunoblotting. (H) PA28γ is required for MDM2-mediated p53 degradation. AS49 cells were transiently transfected with a control siRNA pool (1.25 nM for each duplex) or a PA28γ siRNA pool (1.25 nM for each duplex) to knockdown endogenous PA28γ. Cells were transfected with a plasmid to overexpress MDM2 (3 μg) or a control vector (3 μg) 24 h later. After an additional 24 h, target proteins were detected by immunoblotting. (F) PA28γ and MDM2 exhibit similar kinetics in limiting the accumulation of p53 induced by γ-irradiation. AS49 cells were transfected with plasmids overexpressing MDM2 or PA28γ (3.5 μg each). 24 h later, cells were exposed to 8 Gy γ-irradiation, and whole cell lysates were collected at different time points after irradiation for detection of target proteins. (J) The effect of Nutlin-3 on p53 requires PA28γ. AS49 cells pretreated with the PA28γ siRNA pool (1.25 nM) for each duplex for 12 h) were incubated with 3 μM Nutlin-3 for another 12 h. Target proteins were examined by immunoblotting. (K) p14ARF cannot induce accumulation of p53 when PA28γ is knocked down. HCT116 cells pre-incubated with the PA28γ siRNA pool or control siRNA pool (1.25 nM for each duplex) for 24 h were transfected with plasmids overexpressing p19ARF (3 μg) or a control vector (3 μg). After another 24 h, target proteins in the whole cell lysates were examined by immunoblotting. (L) Daxx loses its capacity to downregulate p53 when PA28γ is absent. MEF cells (PA28γ+/− or PA28γ−/−) were transfected with a plasmid to overexpress Daxx (3 μg) or a control plasmid (3 μg). Whole cell lysates were collected for examination of target proteins 24 h later. (M) PA28γ is not involved in the downregulation of p53 by Y11. HCT116 cells pretreated with the PA28γ siRNA pool or control siRNA pool (1.25 nM for each duplex) for 24 h were transfected with a plasmid to overexpress Y11 (3 μg) or a control plasmid (3 μg). After another 24 h, whole cell lysates were collected for examination of target proteins.
Supporting the specificity of the effect of PA28γ on p53, PA28γ knockdown did not result in apparent changes in the levels of Numb or MDM4 (two other substrates of MDM2) in HCT116 cells, although upregulation of p53 was observed (Figure 5G).

**PA28γ regulates apoptosis through p53**

To demonstrate that PA28γ regulates apoptosis through modulation of p53, we compared survival and apoptosis of cells treated with a PA28γ siRNA pool or a control siRNA pool. The tested cancer cell lines included: A549 (wt p53); a pair of MCF-7 cell lines (one with wt p53 (p53+/+) and one nullizygous for p53 (p53/-/-) by stable knockdown of p53 by siRNA; Li et al, 2005); a pair of HCT116 cell lines (one with wt p53 (p53+/+) and one nullizygous for p53 (p53/-/-) by deletion of p53 through homologous recombination); and PC3 cells (p53 null). Knockdown of PA28γ resulted in a reduction in the survival of A549, MCF-7 (p53+/+), and HCT116 (p53+/+) cells (Figure 6A and Supplementary Figure S7) and in higher numbers of apoptotic cells (Figure 6B and Supplementary Figure S7). In contrast, there were no effects on the survival or apoptosis of MCF-7 p53/C0/C0, HCT116 p53/C0/C0, or PC3 (p53null) cells (Figures 6A and B and Supplementary Figure S7).

Similarly, γ-irradiation-induced accumulation of p53 was reduced by overexpression of PA28γ in MCF-7 cells (Figure 6C), and overexpressed PA28γ stabilized the MDM2–p53 interaction in the presence of γ-irradiation (Figure 6D). The extent of apoptosis in MCF-7 cells with overexpressed PA28γ was also lower than in control cells.
following γ-irradiation (Figure 6E), and knockdown of p53 in HCT116 cells inhibited the radiosensitizing effect of PA28γ siRNA on cells (Figure 6F).

MDM2 is considered a target for human cancer therapy (Bond et al., 2004; Zhang et al., 2005; Poyurovsky and Prives, 2006). Several experimental therapeutic molecules, including a specific anti-MDM2 antisense oligonucleotide (AS), have been developed (Zhang et al., 2003, 2005). However, the response of p53 to inhibition of MDM2 by AS varies in different cell lines. These differences might reflect variations in the basal levels of endogenous PA28γ. Analysis of the basal expression of PA28γ in six cancer cell lines with wild-type p53

**Figure 6: Western blot analysis.**

**A** shows the expression of MDM2, p53, and PA28γ in the cell lines. **B** displays the expression of PA28γ and MDM2. **C** and **D** provide additional information on the interaction between MDM2 and p53. **E** and **F** illustrate the in vitro pull-down assay using GST-MDM2 and p53. **G** presents the elution profile of GFP + p53-HA + MDM2-T7. The elution volume is indicated in ml.
demonstrated that U2OS cells had the lowest level of PA28γ (Figure 6G). Interestingly, MDM2 inhibition in U2OS cells did not result in any appreciable enhancement of the level of p53 (Figure 6H). In contrast, p53 accumulated following inhibition of MDM2 in the other cells (Figure 6H).

To examine whether downregulation of PA28γ enhances the effects of MDM2 inhibition in cancer cells with a high level of PA28γ, HCT116 and A549 cells were exposed to a combination of Nutlin-3 (3 μM) and PA28γ siRNA. The combination led to a synergistic upregulation of p53 (Figure 6I), inhibited survival, and enhanced apoptosis (Figure 6J). This suggests a new strategy for sensitizing human cancers to MDM2 inhibitors.

**Discussion**

The role of MDM2 in regulating p53 is supported by transgenic animal studies (Jones et al, 1995; Montes de Oca Luna et al, 1995). Several regulators of the MDM2–p53 interaction have been identified, including p14ARF (Pomerantz et al, 1998; Zhang et al, 1998a), YY1 (Sui et al, 2004), MDMX (Francoz et al, 2006), gankyrin (Higashitsuji et al, 2005), L11 (Lohrum et al, 2003), and Daxx (Tang et al, 2006). This large number of regulatory molecules indicates the complexity of the MDM2–p53 interaction. The pattern of p53 degradation depends on the cellular level of MDM2 (Li et al, 2003). Further, in different human cancer cell lines, the extent of p53 accumulation varies even when a similar level of MDM2 inhibition has been achieved (Zhang et al, 2005). The identification of PA28γ as a cofactor for the degradation of p53 suggests a new model for p53 degradation, in which PA28γ regulates the MDM2–p53 interaction and MDM2-dependent degradation. Without PA28γ, MDM2 induces p53 monoubiquitination and nuclear export; subsequent polyubiquitination is catalysed by cytoplasmic factors. When PA28γ is present, p53 is efficiently polyubiquitinated by low levels of MDM2 within the nucleus, and the p53 level remains under stringent control by MDM2. This could provide advantages to cells in that it would circumvent the need for elevated levels of MDM2 and the need for nucleus-to-cytoplasm transport.

Recent studies have shown that MDM2 can form polymers to increase the local concentrations of components of the ubiquitination reaction and provide a binding surface for multiple E2s (Poyurovsky et al, 2007). We have observed by FPLC analysis that in lysates of GFP-PA28γ-transfected cells, a portion of MDM2 appeared with GFP-PA28γ and p53 in the fractions with molecular weights higher than 669 kDa (Figure 4G, right panel). It is possible that polymerized PA28γ mediates the interactions not only between p53 and monomeric MDM2 (appearing in fractions ~560 kDa) but also between p53 and polymeric MDM2. This high-order structure would allow for efficient MDM2-dependent polyubiquitination of p53. The protein complex may also provide a scaffold to orient the substrates, in addition to increasing the local concentrations of the components, especially when the amounts of cellular E2 are low.

MDM2 is being considered as a target for human cancer therapy. We have provided evidence that PA28γ has an integral function in regulating the MDM2–p53 interaction by facilitating the interaction of the two proteins. This observation may have clinical implications. In designing future trials of agents targeting MDM2, screening for the expression of PA28γ in cancer patients should be taken into account.

Another interesting observation is that the change in MDM2 expression in different cells is not the same after PA28γ overexpression. MDM2 is a target of p53, and PA28γ is a negative regulator of p53. Therefore, overexpression of PA28γ in cells may result in a decrease in the level of MDM2. This may occur through the inhibition of the transactivation activity of p53 by PA28γ. Although this seems to be the case in A549 cells, at the current stage we cannot completely rule out the possibility that PA28γ may directly regulate MDM2 or may regulate the oncoprotein through other pathways. These mechanisms may be cell-type dependent. Future studies investigating the possible regulatory function of PA28γ on MDM2 expression either in the presence or absence of p53 are warranted.

This study reveals a previously unrecognized mechanism underlying the effects of PA28γ on apoptosis and cell cycle regulation. Although one PA28γ substrate, SRC-3/AIB1, had been identified previously (Li et al, 2006), its pro-growth function in regulating p53 has been revealed.

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**Figure 4** PA28γ protein interacts with both MDM2 and p53 proteins and promotes the MDM2–p53 interaction. (A) Endogenous PA28γ, MDM2, and p53 interact in intact cells. Lysates of MCF-7 cells were immunoprecipitated by PA28γ (Invitrogen) (left panel), MDM2 (N-20) (middle panel), or p53 (FL393) (right panel), and target proteins in the immunocomplexes were separated by SDS-PAGE and detected by antibodies against MDM2 (SMPI4), p53 (DO-1), or PA28γ (BD Biosciences). (B) PA28γ interacts with MDM2 and p53 in vitro. GST-MDM2 (upper panel, left) or GST-PA28γ (lower panel, left) was incubated with in vitro-translated PA28γ or p53. The complex was pulled down by GST beads, and the target proteins were detected by PA28γ or p53 antibody (DO-1). (C) Exogenous MDM2, p53, and PA28γ interact in COS7 cells. COS7 cells were transfected with p53-HA or PA28γ-GFP, or both (upper panel), and cell lysates were immunoprecipitated by GFP or HA antibody. The binding between p53 and PA28γ was examined by immunoblotting with HA or GFP antibody. In a separate experiment, COS7 cells were transfected with MDM2-T7 or PA28γ-GFP, or both (lower panel), then cell lysates were immunoprecipitated by GFP or T7 antibody. The binding between MDM2 and PA28γ was examined by immunoblotting with T7 or GFP antibody. (D-F) PA28γ promotes the binding between endogenous MDM2 and p53 in cells and in vitro. (D) The interaction between MDM2 and p53 in U2OS cells with overexpression of PA28γ (0.3, and 5 μg) (left panel), or in A549 cells with PA28γ knockdown by PA28γ siRNA pool (1.25 nM for each duplex) (right panel), was examined by immunoprecipitation with p53 antibody (FL393) followed by immunoblotting with MDM2 antibody (SMPI4). (E) GST-MDM2 was incubated with in vitro-translated p53 in the presence or absence of PA28γ at 4°C for 4 h. p53 binding by GST-MDM2 was pulled down by GST beads, eluted by SDS sample buffer at 100°C for 10 min, and detected by immunoblotting with p53 antibody, DO-1. (F) Cell lysates of low passage MEF were immunoprecipitated with p53 antibody FL393, and the immunoprecipitates were separated on SDS-PAGE. Target proteins were detected by immunoblotting with specific antibodies. (G) The polymer of PA28γ mediates the interaction with MDM2 and p53. COS7 cells were transfected with plasmids overexpressing GFP or GFP-PA28γ with MDM2-T7 and p53-HA (5 μg for each plasmid per 100 mm dish). Cell lysates were collected and subjected to gel filtration using the Superose 6/Superdex 200 system 24 h later. The absorbance profiles are shown in the left panel. Eluted fractions were subjected to 10% SDS-PAGE, and target proteins were detected by immunoblotting (right panel).
Figure 5 PA28γ specifically downregulates p53. (A–C) PA28γ mutants lacking the capacity to activate the proteasome still promote p53 degradation in cells, whereas a mutant lacking the insert region is incapable of facilitating p53 degradation. U2OS cells were transfected with GFP-PA28γ, mutants P245Y, G150S, N151Y, Δ76–103, or GFP (4 μg for each plasmid). The stability of endogenous p53 was determined by immunoblotting (left panel). The relative densitometry data at each time point are expressed as a percentage of the density of p53 at time 0, normalized to the corresponding β-actin level. The average of the relative densitometry data in the three independent experiments are shown in the right panel. *P < 0.05. (B) U2OS cells were transfected with ubiquitin (2 μg per 60 mm dish) with either GFP, GFP-PA28γ, or GFP-PA28γ-Δ76–103 (4 μg per 60 mm dish) for 28 h, followed by the exposure to proteasome inhibitor MG132 (15 μM) for 4 h. Cell lysates were immunoprecipitated with p53 antibody FL393. The immunoprecipitates were separated on 10% SDS–PAGE and immunoblotted with polyubiquitin chain antibody, FK1. (C) His-p53 (6 ng) was incubated with His-MDM2 (6 ng) and GST-PA28γ (30 ng), or GST-PA28γ-Δ76–103 (30 ng) for 3 h in the presence of E1 (10 ng), E2 (40 ng), and ubiquitin (5 μg). Ubiquitinylated p53 was detected by immunoblotting with the p53 antibody, DO-1. A series of plasmids expressing mutants of PA28γ were transfected with MDM2-Δ76–115 (D) or p53-HA (E) into COS7 cells. Sequential assays of immunoprecipitation with GFP antibody and immunoblotting with T7 (D) or HA (E) antibody were employed to determine the region essential for the binding of PA28γ to MDM2 or p53. (F) A summary of the capacities of the PA28γ mutants to bind to MDM2 and p53. (G) HCT116 cells were treated with a control siRNA duplex or a PA28γ siRNA duplex (10 nM, 48 h). Target proteins were detected by immunoblotting.
nature is difficult to reconcile with the pro-apoptotic and antiproliferative phenotypes following PA28γ knockdown. Recently, p21Waf1 was also demonstrated to be a substrate of PA28γ (Chen et al., 2007; Li et al., 2007). Although the cell cycle arrest phenotype of PA28γ knockdown could be explained by its effects on p21Waf1, the mechanisms responsible for its induction of apoptosis were not clear. We have identified an interaction among PA28γ, MDM2, and p53, which have established activities in apoptosis and cell proliferation. This interaction could provide the missing link between PA28γ and apoptosis.

In support of this theory, PA28γ/C0 mice, although viable, have a phenotype similar to transgenic mice with hypomorphic MDM2. We also observed that PA28γ has a negative regulatory function in the level of p53 in both MEF cells and normal breast epithelial MCF10A cells. It is possible that the viability of PA28γ/C0 mice may depend on the presence of other p53-regulatory molecules when PA28γ is knocked down. For example, we have observed that YY1 is still capable of downregulating p53 in the presence of PA28γ knockdown (Figure 3M). Recent efforts have identified several cellular factors that selectively regulate the transactiva-
tional activities of p53 (Das et al., 2007; Tanaka et al., 2007). These molecules may also regulate the activities of p53 in the absence of PA28γ.

MDM2 knockout results in lethality early in development in mice (Jones et al., 1995; Montes de Oca Luna et al., 1995). This may result from a p53-mediated hypoxic response due to the lack of regulation by MDM2. It is possible that the regulation of MDM2–p53 by PA28γ may not be involved in this stage of early development, or that other cofactors have a more important function at this stage, which could explain the viability of PA28γ−/− mice. Nonetheless, in support of the effects of PA28γ on p53 regulation, PA28γ−/− mice, although viable, have reduced adult body mass, growth retardation, and demonstrate spontaneous apoptosis and G1 arrest in embryonic fibroblasts.

In conclusion, the present study (a) identifies a novel factor involved in the regulation of the MDM2–p53 interaction; (b) indicates that a previously undefined partner of MDM2 is required for the degradation of p53 protein; (c) suggests a novel mechanism by which PA28γ regulates apoptosis and cell proliferation; (d) demonstrates that PA28γ mediates the degradation of intact proteins in addition to peptides; and (e) provides a possible new target for human cancer therapy.

Materials and methods

Cells

LNCaP, A549, MCF-7, and PC3 cells were purchased from the ATCC. U2OS, and HCT116 p53−/− and HCT116 p53+ cells were provided by Dr X Chen (UC Davis) and Dr B Vogelstein (Johns Hopkins), respectively. MCF-7 cells with stable p53 knockdown have been described previously (Li et al., 2005). All cells were cultured under standard conditions described by the ATCC. PA28γ−/− and PA28γ+/− MEFs and immortalized MEFs cells (3 passages after immortalization, ~20 passages from initial culture) were kindly provided by Dr L Barton (Austin College). The temperature-sensitive Balb/c 3T3 cell line, ts20b, was a gift from Dr O Harvey (UMDNJ-New Jersey Medical School).

Antibodies

Antibodies were obtained from Affinity (proteasome lysate/S b subunit, S7/Rpt1, and PA28β), BD Biosciences (mouse PA28γ), Calbiochem (p53 DO-1, T7, and human p21Waf-Ab-1), Cell Signaling (Puma and Numb), Covance (HA.11), Invitrogen (rabbit polyclonal PA28γ), Santa Cruz (p53 FL393, 14-3-3ε, MDM4, MDM2 SMP14, and MDM2 N-20), and Sigma (Flag M2 and GFP-N-terminal).

Plasmids, oligonucleotides, and reagents

Various plasmids were kind gifts from other investigators, including Flag-tagged pcDNA3-PA28γ and pEF-PA28γ (Sigma), pcDNA3-p53-HA, pcGTT7-MDM2, p21Waf luciferase reporter, and pBabe-U6-p53 (Dr X Chen, UC Davis), pcMV-MDM2 and pcMV-p53 (Dr J Chen, Moffitt Cancer Center), HA-UB and YY1-HA (Dr Y Shi, Harvard), and Daxx-HA (Dr X Yang, U Penn). The GFP-C1-PA28γ, PGE2-T2PA28γ, and pcDNA3-PA28γ vectors were generated by hAFLP/PCR and Myc-Ubiquitin for in vitro ubiquitination assays were purchased from Boston Biochem, and 20S and 26S proteasomes were purchased from Biomol.

Immunoprecipitation and immunoblotting

Cells were lysed in NP-40 buffer, then lysates were immunoprecipitated with specific antibodies and examined by immunoblotting. The Supplementary data can be consulted for additional details.

Generation of GST fusion proteins and pull-down assays

Direct physical binding in vitro between the MDM2 protein and PA28γ, or between p53 and PA28γ, was assessed using GST fusion proteins. The bound proteins were captured by glutathione-agarose beads and resolved on SDS–PAGE. Details are available in the Supplementary data section.

Purification of His-tagged recombinant proteins and in vitro ubiquitination assay

MDM2 and p53 fusion proteins were generated in Escherichia coli, then purified on Ni-NTA Superflow Columns. We utilized a previously described protocol to evaluate ubiquitination (Li et al., 2003). Ubiquitinated p53 was detected by immunoblotting with the DO-1 p53 antibody. For more details about these experiments, please see the Supplementary data section.

In vitro proteasome degradation assay

A modification of a published protocol was used to evaluate proteasomal degradation of p53 and p21 (Dai et al., 2006; see Supplementary data). An aliquot of the reaction was analysed by immunoblotting.

Figure 6 PA28γ regulates apoptosis through p53. (A, B) PA28γ knockdown results in p53–mediated cell killing. (A) A549, MCF-7 p53+/−, and MCF-7 p53−/− cells were treated with a control siRNA pool (5 nM of each duplex) or a PA28γ siRNA pool (1.25, 2.5, or 5 nM of each duplex), and cell viability was determined by MTT assay. (B) A549, MCF-7 p53+/−, and MCF-7 p53−/− cells were treated with a PA28γ siRNA pool or control siRNA pool as above, and apoptotic cells were detected by flow cytometry. The relative percentages of surviving cells and apoptotic indices are presented as the means ± s.d. of three independent samples. (C–F) PA28γ inhibits p53 accumulation and apoptosis following γ-irradiation (IR). (C) MCF-7 cells were transfected with PA28γ (3 μg) or control vector (3 μg), 24 h later cells were exposed to γ-irradiation (8 Gy). Target proteins in whole cell lysates collected at different time points after irradiation were detected by immunoblotting. (D) A549 cells were transfected with a plasmid overexpressing PA28γ (3 μg) or the control vector (3 μg). Cells were exposed to γ-irradiation (8 Gy) 24 h later. After different time periods, cells lysates were immunoprecipitated by p53 antibody FL593, and the immunoprecipitates were separated on 10% SDS–PAGE. Target proteins were detected by immunoblotting. (E) MCF-7 cells with transiently overexpressed GFP-PA28γ (3 μg) or GFP (3 μg) were exposed to γ-irradiation. After 8 h, cells were collected and fixed in 70% ethanol at 4°C overnight. After staining with PI, GFP-positive cell populations were counted by flow cytometry to determine DNA content, and the sub-G1 populations were selected to represent the apoptotic cells. Relative apoptotic indices are presented as the means ± s.d. of three independent samples. (F) HCT116 p53+/− and HCT116 p53−/− cells were pretreated with the PA28γ siRNA or control siRNA pool (0.625 nM for each siRNA) for 24 h or were exposed to γ-irradiation (8 Gy). After another 24 h, cell viability was determined by MTT assay. The relative percentages of surviving cells are presented as means ± s.d. of three independent samples. (G, H) The enhanced levels of p53 in different cancer cell lines upon MDM2 inhibition is related to the level of endogenous PA28γ. Cancer cells were examined for basal PA28γ protein (G), then treated with MDM2 AS (50 and 150 nM) or ASM (150 nM). After 24 h, target proteins were detected by immunoblotting (H). (I, J) PA28γ knockdown in human cancer cells with high levels of endogenous PA28γ sensitizes them to Nutlin-3. (I) HCT116 and A549 cells were transfected with a control or PA28γ siRNA pool (1.25 nM for each duplex). 3 h after transfection, 3 μM Nutlin-3 or the solvent, DMSO, was added to these media, and 24 h later, target proteins were detected by immunoblotting. (J) A549 cells were treated as described in (G), then cell viability was determined by MTT assay, and apoptotic cells were detected by flow cytometry. The relative percentages of surviving cells and apoptotic indices are presented as the means ± s.d. of three independent samples. MTT and apoptosis assays were performed at least twice, and similar results were observed. *P < 0.05; **P < 0.0001.
siRNA transfection
The PA28γ, p53, and control siRNA pools, individual siRNA duplexes targeting PA28γ or PA28β, the control siRNA duplex randomly selected from the siRNA pool, and the transfection reagent DharmaFECT™1 were purchased from Dharmacon. siRNA transfection was performed according to the manufacturer’s protocol. For MTT and apoptosis assays using the siRNA pool, cells were incubated with 1.25 nM of each siRNA for 24 h; for MTT and apoptosis assays, 0.625, 1.25, 2.5, or 5 nM of each siRNA was incubated with cells for 48 h.

Analytical gel filtration chromatography
Analysis of protein profiles of lysates from COS7 cells transfected with MDM2-T7, p53-HA, GFP, or GFP-PA28 by the FPLC method was accomplished following a previously described protocol (Garber et al., 2000). Detailed information is provided in the Supplementary data.

RT–PCR
The extraction of total RNA, reverse transcription, and PCR were performed according to the procedures described previously (Zhang et al., 2005). The extraction of total RNA, reverse transcription, and PCR were performed according to the procedures described previously (Zhang et al., 2003). Details are available in the Supplementary data.

Luciferase assay
Cells were co-transfected with full-length p21WAF1 promoter vectors with the Renilla luciferase reporter (as an internal control; Promega) for 24 h. The luciferase activity of the p21WAF1 promoter reporter was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the provided protocol, and normalized to the corresponding Renilla luciferase reporter activity.

Cell survival, apoptosis, and cell cycle distribution assays
MTTs assay to measure the cell viability, and apoptosis and cell cycle distribution assays were accomplished according to methods described previously (Li et al., 2005).

γ-Irradiation
Cells were placed in a 60Co Picker unit irradiator (1.56 Gy/min) and exposed to 8.0 Gy γ-irradiation.

Statistical analysis
The means of relative densitometry of p53 bands, survival indices, and apoptotic indices between two treatment groups were analysed using the two-tailed paired t-test. The significance of the variances of means of indices between treatment groups was initially analysed by ANOVA. If positive (P<0.05), the means between the control and each treatment group were analysed using the two-tailed paired t-test.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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