Association of p19^{ARF} with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53

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We have demonstrated previously that the oncoprotein Mdm2 has a ubiquitin ligase activity for the tumor suppressor p53 protein. In the present study, we characterize this ubiquitin ligase activity of Mdm2. We first demonstrate the ubiquitination of several p53 point mutants and deletion mutants by Mdm2. The point mutants, which cannot bind to Mdm2, are not ubiquitinated by Mdm2. The ubiquitination of the C-terminal deletion mutants, which contain so-called Mdm2-binding sites, is markedly decreased, compared with that of wild-type p53. The binding of Mdm2 to p53 is essential for ubiquitination, but p53's tertiary structure and/or C-terminal region may also be important for this reaction. DNA-dependent protein kinase is known to phosphorylate p53 on Mdm2-binding sites, where DNA damage induces phosphorylation, and p53 phosphorylated by this kinase is not a good substrate for Mdm2. This suggests that DNA damage-induced phosphorylation stabilizes p53 by inhibiting its ubiquitination by Mdm2. We further investigated whether the tumor suppressor p19^{ARF} affects the ubiquitin ligase activity of Mdm2 for p53. The activity of p19^{ARF}-bound Mdm2 was found to be lower than that of free Mdm2, suggesting that p19^{ARF} promotes the stabilization of p53 by inactivating Mdm2.

Keywords: DNA-PK/Mdm2/p19^{ARF}/p53/ubiquitin

Introduction

The tumor suppressor p53 binds to specific sequences on the promoter regions of target genes and functions as a transcriptional factor, inducing the expression of genes such as those for GADD45 (Kastan *et al.*, 1992; Lu and Lane, 1993), WAF1/p21/CIP1 (El-Deiry *et al.*, 1993), cyclin G (Okamoto and Beach, 1994), Bax (Miyashita and Reed, 1995), IGF-BP3 (Buckbinder, *et al.*, 1995), 14-3-3 σ (Hermeking *et al.*, 1998) and Mdm2 (Barak *et al.*, 1993) (for reviews, see Gottlieb and Oren, 1996; Ko and Prives, 1996). These gene products regulate cell-cycle arrest and apoptosis. The expression of these genes is induced immediately after induction of p53, except for Mdm2 (for a review, see Levine, 1997), whose expression does not increase until at least 1 h after the induction of p53.

In normal cells, p53 is maintained at very low, often undetectable levels. In response to various types of genotoxic stress, however, the level of p53 increases (for

reviews, see Gottlieb and Oren, 1996; Ko and Prives, 1996). Both the inhibition of its degradation and activation of translation have been thought to be important mechanisms for this increment. The degradation of p53 is regulated by the ubiquitin–proteasome pathway (Maki *et al.*, 1996). In this pathway, a ubiquitin–protein adduct is formed by three sequential steps (for a review, see Ciechanover, 1994) which involve the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3). E1 is a common enzyme in all ubiquitination reactions, and the specificity for the targeted protein is dependent on E2 and E3 (Huibregtse *et al.*, 1995; Sheffner *et al.*, 1995). The resultant polyubiquitinated proteins are degraded by the proteasome.

The *Mdm2* gene was found to be amplified in 30–40% of human sarcomas (Oliner *et al.*, 1992), where the p53 gene was not mutated. Mdm2 protein has the ability to bind to p53 and has been thought to suppress the transcriptional factor activity of p53 by interacting with its N-terminal region transcriptional activation domain (Oliner *et al.*, 1993; Wu *et al.*, 1993). DNA-dependent protein kinase (DNA-PK) phosphorylates serine and threonine residues in the N-terminal region of p53, where DNA damage induces phosphorylation *in vivo*, and this phosphorylation reduces the interaction of p53 with Mdm2 (Shieh *et al.*, 1997). When Mdm2 cDNA is transfected into human cells, p53 degradation is accelerated (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). In agreement with these data, we reported that Mdm2 protein functions as an E3 ubiquitin ligase, using UbcH5 as E2, for the p53 *in vitro* ubiquitination system (Honda *et al.*, 1997).

The *INK4a/ARF* gene has two promoters and it encodes two completely different proteins, p16^{INK4a} and p19^{ARF} (Ouelle *et al.*, 1995; for a review, see Larsen, 1997). p16^{INK4a} binds to cyclin D–CDK4 and –CDK6 complexes and inhibits their kinase activities. This inhibition decreases the phosphorylation of Rb protein, resulting in cell-cycle arrest at G₁. The other encoded protein, p19^{ARF}, does not bind to any cyclin–CDKs but seems to have a tumor suppression function dependent upon the presence of wild-type p53 (Kamijo *et al.*, 1997; de Stanchina *et al.*, 1998; Pomerantz *et al.*, 1998; Zhang *et al.*, 1998; Zindy *et al.*, 1998). p19^{ARF} was shown to bind to Mdm2 (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998) and its association was shown to stimulate the degradation of Mdm2 (Zhang *et al.*, 1998). Furthermore, p19^{ARF} interacts directly with the p53–DNA complex (Kamijo *et al.*, 1998).

Here we show that p19^{ARF} inhibits the ubiquitin ligase activity of Mdm2 and that p53 phosphorylated by DNA-PK is not a good substrate for Mdm2. Furthermore, the C-terminal region of p53 is shown to be important for Mdm2-induced ubiquitination.

Fig. 1. Mdm2-dependent ubiquitination of p53 *in vitro*. Lanes 1–3: Sf-9 cell extract expressing GST-p53 was incubated with E1, E2 (UbcH5), biotinylated ubiquitin and Sf-9 cell extract expressing His-Mdm2 in 50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 2 mM ATP and 2 mM DTT at 25°C for 30 min. Then GST-p53 was pulled down by glutathione-Sepharose 4B. Purified p53 was resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was reacted with ExtraAvidin peroxidase at room temperature for 1 h. Ubiquitinated p53 was visualized by ECL; lanes 4–6, ubiquitinated p53 from lanes 1–3 was detected by anti-p53 antibody (C-19); lanes 7–9, His-p53 and GST-Mdm2 were used and purified by immunoprecipitation with anti-p53 antibody (PAb1801), followed by ECL detection of ubiquitination as described for lanes 1–3.

Results

Ubiquitination of wild-type p53 by Mdm2

In our previous study, we detected the ubiquitination of p53 by using biotinylated ubiquitin. Human p53, which was fused to glutathione *S*-transferase (GST) N-terminally, was ubiquitinated by human Mdm2 (hdm2) in the presence of an E1, E2 (UbcH5) and biotinylated ubiquitin *in vitro* ubiquitination system (Honda *et al.*, 1997; Figure 1, lanes 1–3). We tested the ubiquitinated proteins shown in Figure 1, lanes 1–3, and detected the ladder of bands by immunoblotting using anti-p53 antibody (Figure 1, lanes 4–6). In a standard assay, GST-p53 was purified by glutathione-Sepharose 4B after the ubiquitination reaction. When His-tagged p53 was used as substrate, p53 protein was purified by immunoprecipitation. As shown in Figure 1, lanes 7–9, similar results were obtained using His-tagged p53 as substrate. The ubiquitination of His-tagged (data not shown) or untagged p53 was also detected by immunoblotting (Figure 4A).

Although the reaction mixture does not contain Mdm2, we could detect mono- or weaker di-ubiquitinated p53 bands (Figure 1, lanes 4 and 7). This result may be due to the presence of proteins originating from the Sf-9 cell extract containing p53, because we did not detect such a ubiquitinated protein band using purified p53 (Honda *et al.*, 1997; Figure 4B).

Ubiquitination of mutant p53 proteins by Mdm2

To study further the Mdm2-mediated ubiquitination of p53, we constructed a series of C-terminal truncation mutants of p53 and tested them for ubiquitination by Mdm2. All truncated forms showed the expected molecular sizes on SDS-PAGE (Figure 2A, upper panel). The level of ubiquitination of the truncation mutants was dramatically decreased relative to that of full-length p53 (1–393 residues) (Figure 2B). Even when only 23 residues were deleted, the deletion (1–369) strikingly affected Mdm2-mediated ubiquitination. A previous study showed that p53 interacts with Mdm2 via its N-terminal region, and point mutations in this region inhibit their interaction. We found that the two types of double point mutants

(L14Q/F19C and L22Q/W23S) failed to interact with Mdm2 *in vitro* (Figure 2C, lanes 2 and 3) and were not ubiquitinated by Mdm2 (Figure 2D, lanes 1–4). Although the truncated forms contained the Mdm2-binding site in the N-terminal region of p53, the mutants (1–289 and 1–328) did not bind to Mdm2. Although the mutant (1–369) bound to Mdm2 only slightly more weakly than wild-type p53, its level of ubiquitination was much lower. These data suggest that interaction between p53 and Mdm2 is required for p53 ubiquitination, but it is not sufficient. We speculate that the ubiquitination of p53 by Mdm2 may need a strict conformation, for which the C-terminal region may be important.

Arg249 in human p53 is one of the ‘hot spots’, often mutated in tumor cells. We next assessed the effect of mutation at Arg249 on the ubiquitination. Substitution by Ser (R249S), which is detected in tumor cells, hardly affected the level of ubiquitination, whereas R249A showed a slight decrease in ubiquitination (Figure 2D). The ability to bind to Mdm2 was not different in either mutant (Figure 2C). These data showed that a tumor-derived mutation had no effect on this ubiquitination system; however, we did not test other mutants detected in tumors. These results are summarized in Figure 2E.

Effect of phosphorylation of p53 by DNA-PK on ubiquitination

p53 is phosphorylated after γ or UV irradiation and one of the phosphorylated residues is assigned as Ser15, which is important for Mdm2 binding. Ser15 and Ser37 are also phosphorylated *in vitro* by DNA-PK (Prives, 1998). We tested the effect of phosphorylation within the N-terminal region of p53 on Mdm2-induced ubiquitination. As expected, recombinant GST-p53 was phosphorylated by DNA-PK in the presence of exogenous DNA *in vitro* (Figure 3A and C). After the kinase reaction, GST-p53 was bound to glutathione-Sepharose 4B and precipitated by centrifugation. The GST-p53 was then used for the ubiquitination reaction. The addition of exogenous DNA greatly reduced the level of p53 ubiquitination (Figure 3B, lanes 1–3). This may be due to DNA binding directly to p53, because the ubiquitination ability was recovered when DNA was washed out with buffer containing 0.4 M NaCl (compare lanes 1 and 4). After washing with salt-containing buffer, non-phosphorylated p53 was poly-ubiquitinated normally, but the phosphorylated p53 was ubiquitinated to a lesser extent (Figure 3B, lanes 4–6). Phosphorylation by DNA-PK caused a reduction in p53 ubiquitination (Figure 3B and C, lanes 5 and 6), whereas p53 phosphorylated by casein kinase I (CKI) was ubiquitinated to the same extent as the non-phosphorylated form (Figure 3C, lanes 7 and 8). We used CKI as a phosphorylation control; its phosphorylation site has not been identified yet. It has been shown that CKI phosphorylates the N-terminal portion of p53 *in vitro* (Ko and Prives, 1996).

Effect of p19^{ARF} on Mdm2 activity

Recently it has been shown that the tumor suppressor p19^{ARF} can prevent p53 degradation, which is involved in binding of p19^{ARF} to Mdm2 (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). We therefore assessed the ubiquitination activity of p19^{ARF}-bound Mdm2. His-Mdm2 bound to GST-p19^{ARF} was obtained by pull-down with

glutathione–Sepharose 4B after mixing cell extracts containing these proteins. Because we used GST-fused Mdm2 as a positive control, p19^{ARF}-binding His-Mdm2 (ARF/MDM2) and non-binding GST–Mdm2 (MDM2) showed different migrations on SDS–PAGE (Figure 4A and B, lanes 7 and 8, and C). These proteins, either bound to glutathione–Sepharose 4B resin or eluted from the resin, were used in Figure 4A and B, respectively. In Figure 4A, we performed a ubiquitination assay using Sf-9 cell extract expressing untagged p53 as substrate and detected the ladder of p53 by immunoblotting. The level of p53 ubiquitination by p19^{ARF}-bound Mdm2 apparently

decreased, relative to the non-binding form (Figure 4A, lanes 1–6). When purified proteins, p19^{ARF}-bound Mdm2 (ARF/MDM2), free Mdm2 (MDM2) and GST–p53, were used in the reaction, free Mdm2 had ubiquitin ligase activity but p19^{ARF}-bound Mdm2 did not (Figure 4B). We detected the background level of ubiquitination in Figure 4A in the presence of only UbcH5, but not when affinity-purified p53 was used as substrate. In these experiments, the amounts of Mdm2 added to the reaction were quantified by immunoblotting using anti-Mdm2 antibody and they were almost equal in p19^{ARF}-bound Mdm2 and free Mdm2 (Figure 4A and B, lanes 7 and 8). Although we compared the ubiquitination activity of His-Mdm2 with GST–Mdm2, there was no difference in activity (data not shown).

These data indicate that p19^{ARF} inhibits the ubiquitination activity of Mdm2. In support of this, we showed that the auto-ubiquitination ability of p19^{ARF}-bound Mdm2 was much less than that of free Mdm2 (Figure 4C). In the presence of UbcH5, free Mdm2 protein seemed to disappear and it migrated more slowly on SDS–PAGE (see the long exposure in Figure 4C). We confirmed that these were ubiquitinated Mdm2 proteins by detecting biotinylated ubiquitin (data not shown). p19^{ARF}-bound Mdm2 itself was also slightly ubiquitinated (see long exposure), but the ubiquitination was very low. Both p19^{ARF} and phosphorylation of p53 by DNA-PK affect the ubiquitination of p53 through Mdm2, resulting in stabilization of p53.

Discussion

The mechanism by which p53 is increased after γ or UV irradiation has not been clarified yet. The mechanism includes both activation of translational initiation and inhibition of degradation of p53, but does not include transcriptional activation of the p53 gene. The ubiquitin–proteasome system is thought to be responsible for the stabilization of p53 after irradiation, because the poly-ubiquitinated forms of p53 were decreased after irradiation. The ubiquitination of p53 was catalyzed by three steps of enzyme reaction. These enzymes were named E1, E2 and

Fig. 2. Ubiquitination by Mdm2 in relation to the structure of p53. (A) Interaction of p53 C-terminal truncation mutants with Mdm2. GST-fused p53 truncation mutants were incubated with His-Mdm2 at 4°C for 30 min. After incubation, p53 mutants were pulled down with glutathione–Sepharose 4B. Mdm2 was detected by immunoblotting using anti-Mdm2 antibody (SMP14) (lower panel). The upper panel shows membrane staining by amidoblack. An almost equal amount of p53 protein was detected. Lane 1, amino acids 1–160; lane 2, amino acids 1–289; lane 3, amino acids 1–328; lane 4, amino acids 1–350; lane 5, amino acids 1–369; lane 6, amino acids 1–393 (wild-type). (B) *In vitro* ubiquitination of p53 truncation mutants by Mdm2 was performed as described in Figure 1. (C) Interaction of p53 containing the point mutations (L14Q/F19C, L22Q/W23S, R249A and R249S) with Mdm2. The binding assay was performed as described in (A). Upper panel, staining of membrane by amidoblack; lower panel, immunoblotting by anti-Mdm2 antibody. Lane 1, wild-type; lane 2, L14Q/F19C; lane 3, L22Q/W23S; lane 4, R249A; lane 5, R249S. (D) Ubiquitination of p53 mutants described in (C) by Mdm2. Lanes 1 and 2, L14Q/F19C; lanes 3 and 4, L22Q/W23S; lanes 5 and 6, R249S; lanes 7 and 8, R249A; lanes 9 and 10, wild-type. Lanes 1, 3, 5, 7 and 9, Mdm2 (–); lanes 2, 4, 6, 8 and 10, Mdm2 (+). (E) Schematic representation of p53 and its interaction and ubiquitination activity.

Fig. 3. Effect of p53 phosphorylation by DNA-PK on Mdm2-mediated ubiquitination. **(A)** Phosphorylation of p53 by DNA-PK. GST-p53 bound to glutathione-Sepharose 4B was incubated with (lanes 2 and 3) or without (lane 1) 20 U of DNA-PK in the presence (lanes 1 and 2) or absence (lane 3) of DNA at 30°C for 15 min. After incubation, GST-p53 was pulled down by centrifugation and washed. p53 protein was detected by amidoblack staining. Phosphorylated p53 migrated more slowly than the non-phosphorylated form on an SDS-polyacrylamide gel. **(B)** Ubiquitination of p53 phosphorylated by DNA-PK. The kinase reaction was performed as described in (A). Purified GST-p53 was washed three times with the buffer (10 mM Tris-HCl pH 7.4, 3 mM MgCl₂, 0.1 mM PMSF and 0.01% Brij 35) containing (lanes 4–6) or not containing (lanes 1–3) 0.4 M NaCl. The ubiquitination assay was performed as described in Figure 1. Lanes 1 and 4, DNA-PK(-); lanes 2, 3, 5 and 6, DNA-PK(+); lanes 1, 2, 4 and 5, DNA(+); lanes 3 and 6, DNA(-). **(C)** Inhibitory effect of DNA-PK, but not CKI, on p53 ubiquitination. Lanes 1–4 showed the ³²P incorporation of p53 after the kinase assay using 20 U of DNA-PK or CKI. After incubation, GST-p53 bound to glutathione-Sepharose 4B was washed with the 0.4 M NaCl buffer and the ubiquitination assay was performed (lanes 5–8). Lanes 1 and 5, DNA-PK(-), DNA(+); lanes 2 and 6, DNA-PK(+), DNA(+); lanes 3 and 7, CKI(-); lanes 4 and 8, CKI(+).

E3. The key enzyme of the substrate recognition is E3, ubiquitin ligase. The ubiquitin ligase for p53 is shown to be Mdm2 in normal cells (Honda *et al.*, 1997) and E6/E6-AP in papilloma virus-infected cells (Huibregtse *et al.*, 1993; Scheffner *et al.*, 1993; Kumar *et al.*, 1997). The 26S proteasome can only degrade a polyubiquitinated protein which is more than tetra-ubiquitinated (Ciechanover *et al.*, 1998). Mono- and di-ubiquitinated proteins are not recognized as substrates in the ubiquitin-proteasome system. Using an *in vitro* ubiquitination assay, we often detect mono-ubiquitinated protein, which does not seem to be important for proteolysis (Figures 1, 2 and 4).

Wild-type p53 proteins are maintained at a low level in normal cells, whereas mutant p53 proteins are maintained at a high level in tumor cells. As shown in Figure 2, the binding of p53 to Mdm2 is essential for the ubiquitination. A mutation (R249S) of p53, which occurs in many kinds of tumor cells (for a review, see Ko and

Fig. 4. Decrease in ubiquitination activity of Mdm2 by interaction with p19^{ARF}. **(A)** GST-Mdm2 (MDM2) and GST-p19^{ARF}/His-Mdm2 (ARF/MDM2) were purified by glutathione-Sepharose 4B. The untagged p53 expressed in an Sf-9 cell extract was used as substrate and the reaction was performed as described in Figure 1. After incubation, the reaction was stopped by addition of SDS sample buffer and boiled. The ubiquitination ladder of p53 was visualized by immunoblotting using anti-p53 antibody (C-19) (lanes 1–6). **(B)** Using purified GST-p53, GST-p19^{ARF}/Mdm2 and GST-Mdm2, the ubiquitination assay was performed. In brief, GST-p19^{ARF}/Mdm2 and GST-Mdm2 were precipitated by glutathione-Sepharose 4B and eluted with 2 mM glutathione and 50 mM Tris-HCl pH 8.0, followed by incubation with GST-p53-conjugated resin, E1, UbcH5 and biotinylated ubiquitin. Ubiquitinated p53 was detected by horseradish peroxidase-conjugated avidin (lanes 1–6). **(C)** Auto-ubiquitination activity of Mdm2. Mdm2 from the reaction shown in (A) was detected by immunoblotting using anti-Mdm2 antibody (SMP14). Lanes 1–3, ARF/Mdm2; lanes 4–6, Mdm2. Lanes 1, 2, 4 and 5, UbcH5(+); lanes 3 and 6, UbcH5(-). Lanes 2, 3, 5 and 6, Mdm2(+); lanes 1 and 4, Mdm2(-). The amounts of Mdm2 in the reaction were compared by immunoblotting (lanes 7 and 8 in A and B). His-Mdm2 was detected in lane 7 in (A and B), and in lanes 2 and 3 in (C). GST-Mdm2 was detected in lane 8 in (A and B), and in lanes 5 and 6 in (C).

Prives, 1996), does not affect the association with Mdm2 and the mutant is not ubiquitinated at lower levels than the wild-type. These data indicate that accumulation of mutant protein is not due to poor ubiquitination. The transcription of the *Mdm2* gene is activated by p53, and the mutant p53, R249S, cannot activate the transcription. Thus, in tumor cells which have the mutant p53, the level of the Mdm2 protein could be too low to ubiquitinate the

mutant p53, resulting in p53 accumulation. The conformation of p53 is also likely to be important for Mdm2-mediated ubiquitination. Even though it could still bind to Mdm2, the ubiquitination level of a C-terminal truncation mutant (1–369 residues) was markedly reduced. The C-terminal 30 residues of p53 have been shown to regulate the specific DNA-binding function of the central core domain. This region contains the protein kinase C and CKII phosphorylation sites, and the p300 acetylation sites. How the region functions in ubiquitination remains to be clarified.

It is likely that there are multiple pathways for activation of p53 in cells. γ or UV irradiation can induce p53 accumulation, which is partly due to inhibition of protein degradation. p53 is phosphorylated on its N-terminal region in response to irradiation (for a review, see Gottlieb and Oren, 1996; Ko and Prives, 1996). After DNA damage, there is a reduced interaction of p53 with Mdm2 *in vivo* (Shieh *et al.*, 1997). Also, phosphorylation of p53 at Ser15 and Ser37 by DNA-PK reduces the binding activity of Mdm2. We show here that p53 phosphorylated by DNA-PK is no longer a good substrate for Mdm2-mediated ubiquitination (Figure 3). These data suggest that the stabilization of p53 is partly attributable to phosphorylation of p53 by DNA-PK. Recently it has been reported that ATM, which is induced by ionizing radiation, also phosphorylates p53 on Ser15 (Banin *et al.*, 1998; Canman *et al.*, 1998). There may be various protein kinases which are activated by different types of stress and whose functions are similar.

p19^{ARF} could interact with Mdm2, and p53 also binds p19^{ARF} through Mdm2 (data not shown). Previous studies showed that the tumor suppressor p19^{ARF} can prevent p53 degradation through the binding of p19^{ARF} to Mdm2 and showed that this is due to acceleration of Mdm2 degradation by p19^{ARF} association (Zhang *et al.*, 1998). We did not test the effect of p19^{ARF} on Mdm2 degradation. However, our results indicate that the interaction of p19^{ARF} with Mdm2 inhibits the ubiquitin ligase activity of Mdm2. As shown in Figure 4C, p19^{ARF}-bound Mdm2 is barely ubiquitinated; thus if p19^{ARF} accelerates Mdm2 degradation, it is possible that p19^{ARF}-bound Mdm2 could be degraded not by a ubiquitin–proteasome system, but by some other proteolytic system. When p19^{ARF} cDNA was transfected into HeLa cells, the ubiquitination of p53 was hardly detected any more (Pomerantz *et al.*, 1998). This result is coincident to our *in vitro* data shown here.

Even though it is not genotoxic stress, hypoxia treatment has also been shown to stabilize p53. The stabilization requires transcriptional factor HIF-1 α (hypoxia-inducible factor), which is accumulated by hypoxia (An *et al.*, 1998). A mechanism for p53 stabilization by HIF-1 α has not been clarified yet, but this stabilization may also be involved in Mdm2-mediated ubiquitination.

Materials and methods

cDNA cloning

Human Mdm2 (Oliner *et al.*, 1992) and UbcH5 (Sheffner *et al.*, 1994) cDNAs were obtained by RT–PCR using total RNA from HeLa S3 cells. Mouse p19^{ARF} was obtained by RT–PCR using total RNA from mouse testis. The mutant p53 cDNAs were prepared by PCR using a primer containing the mutated base(s).

Expression and preparation of proteins

UbcH5 was ligated into the pET3 vector and expressed in *Escherichia coli* BL 21. After *E.coli* was disrupted by sonication using a buffer containing 10 mM Tris–HCl pH 8.0, 1.0% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.15 M NaCl, UbcH5 was purified by ammonium sulfate precipitation, followed by chromatography using MonoS (Pharmacia). Baculovirus-expressed mouse E1 (Imai *et al.*, 1992) protein was purified by a ubiquitin affinity column.

Human p53, human Mdm2 and mouse p19^{ARF} were inserted N-terminally into GST-fused or His-tagged pFastBac vector (Gibco-BRL) and the proteins were expressed in Sf-9 cells according to the manufacturer's protocol. Sf-9 cells expressing recombinant protein were suspended in 10 mM Tris–HCl pH 7.4, 3 mM MgCl₂, 1 mM PMSF and 0.1% NP-40 at a final concentration of 4×10^6 cells/ml, then briefly sonicated using Handy Sonic UR-20P (Tomy Seiko). The supernatant, after centrifugation at 14 000 g for 10 min, was used for the ubiquitination reaction and *in vitro* binding assay. In some experiments, GST–p53 and GST–Mdm2 were purified by using glutathione–Sepharose 4B (Pharmacia). To obtain His–Mdm2 bound to GST–p19^{ARF}, cell extract containing Mdm2 was incubated with extract containing p19^{ARF} at 4°C for 30 min and purified by glutathione–Sepharose 4B (Pharmacia). GST–p19^{ARF}-bound Mdm2 was eluted with 2 mM reduced glutathione and 50 mM Tris–HCl pH 8.0. GST–Mdm2 was obtained by elution from resin under the same conditions.

In vitro ubiquitination assay

Ubiquitin (Sigma) was biotinylated using EZ-Link sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer's protocol and unreacted biotin was removed by use of NAP-10 (Pharmacia) exclusion chromatography as described previously (Funabiki *et al.*, 1997; Honda *et al.*, 1997; Nakajima *et al.*, 1998). The standard reaction mixture (50 μ l) contained Sf-9 cell extract expressing GST–p53 (500 ng), mouse E1 (500 ng), UbcH5 (500 ng), biotinylated ubiquitin (15 μ g), and cell extract expressing His–Mdm2 (500 ng), 50 mM Tris–HCl pH 7.4, 5 mM MgCl₂, 2 mM ATP and 2 mM dithiothreitol (DTT). After incubation at 25°C for 30 min, GST–p53 was pulled down with glutathione–Sepharose 4B (Pharmacia) and resolved by 7.5% SDS–PAGE. The proteins in the gel were transferred to a PVDF membrane (Millipore). The membrane was incubated with ExtrAvidin peroxidase (Sigma) at room temperature for 1 h and washed. Then the ubiquitinated proteins were visualized by ECL (Amersham). In some experiments, the PVDF membrane was immunoblotted with anti-p53 antibody (C-19, Santa Cruz) or anti-Mdm2 antibody (SMP14, Santa Cruz), followed by the reaction with secondary antibody, peroxidase-conjugated anti-goat IgG or anti-mouse IgG, respectively. The proteins which reacted with the antibody were detected by ECL (Amersham).

In some cases, glutathione–Sepharose 4B-conjugated GST–p53 was added to the ubiquitination reaction mixture and recovered by centrifugation after incubation.

Phosphorylation of p53 by DNA-PK or casein kinase I

GST–p53 bound to glutathione–Sepharose 4B was incubated with 20 U of DNA-PK (Promega) in the presence of 20 ng/ μ l of calf thymus DNA in 20 mM Tris–HCl pH 7.4, 5 mM MgCl₂, 50 mM KCl, 1 mM DTT and 0.1 mM ATP with or without [γ -³²P]ATP at 30°C for 15 min. After incubation, GST–p53 was pulled down by centrifugation and washed with buffer (0.4 M NaCl, 10 mM Tris–HCl pH 7.4, 3 mM MgCl₂, 0.1 mM PMSF and 0.01% Brij 35), followed by the ubiquitination reaction as described above. When p53 was phosphorylated by CKI, p53 was incubated with 20 U of CKI (Promega) in 25 mM Tris–HCl pH 7.4, 10 mM MgCl₂, 0.1 mM ATP and [γ -³²P]ATP.

In vitro binding assay

Sf-9 cells expressing GST–p53 (wild-type or mutant) and His–Mdm2 were suspended in lysis buffer (10 mM Tris–HCl pH 7.4, 3 mM MgCl₂, 0.1 mM PMSF and 0.1% NP-40) and disrupted by brief sonication. After centrifugation at 14 000 g for 10 min, cell extract was obtained. Cell extract containing wild-type or mutant p53 was incubated with the extract containing Mdm2 at 4°C for 30 min. GST–p53 was recovered by glutathione–Sepharose 4B (Pharmacia) and washed with lysis buffer containing 0.01% Brij 35 three times. The precipitates were electrophoresed on a 7.5% SDS–polyacrylamide gel and transferred to a PVDF membrane. Mdm2 was detected by immunoblotting using anti-Mdm2 antibody (SMP14, Santa Cruz).

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