Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1

Monica Gostissa[1], Arnd Hengstermann[2], Valentina Fogal[1], Peter Sandy[1], Sylvia E.Schwarz[2], Martin Scheffner[2] and Giannino Del Sal[1,3,4]

[1]Laboratorio Nazionale, Consorzio Interuniversitario Biotecnologie, AREA Science Park, Padriciano 99, 34012 Trieste, [3]Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università degli Studi di Trieste, Via L. Giorgeri 1, 34100 Trieste, Italy and [2]Deutsches Krebsforschungszentrum, Angewandte Tumorvirologie, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany

[4]Corresponding author
e-mail: delsal@sci.area.trieste.it

The growth-suppressive properties of p53 are controlled by posttranslational modifications and by regulation of its turnover rate. Here we show that p53 can be modified *in vitro* and *in vivo* by conjugation to the small ubiquitin-like protein SUMO-1. A lysine residue at amino acid position 386 of p53 is required for this previously undescribed modification, strongly suggesting that this lysine residue serves as the major attachment site for SUMO-1. Unlike ubiquitin, attachment of SUMO-1 does not appear to target proteins for rapid degradation but rather, has been proposed to change the ability of the modified protein to interact with other cellular proteins. Accordingly, we provide evidence that conjugation of SUMO-1 to wild-type p53 results in an increased transactivation ability of p53. We suggest that posttranslational modification of p53 by SUMO-1 conjugation provides a novel mechanism to regulate p53 activity.
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Introduction

The development of neoplasia correlates with functional inactivation of the p53 tumor suppressor in half of the human cancers (Hollstein *et al.*, 1996). p53 is a transcription factor that can inhibit cell cycle progression and/or induce apoptosis. Loss of wild-type (wt) p53 function, therefore, leads to deregulated cell proliferation, resulting in genomic instability and eventually in the development of malignant lesions (Ko and Prives, 1996; Levine, 1997).

In normal cells, p53 has a short half-life and is expressed at low levels. Furthermore, it is maintained in a latent form but rapidly accumulates and is activated under various stress conditions, including DNA damage, ribonucleotide depletion or generation of reactive oxygen species. The exact mechanism(s) mediating p53 response to these environmental signals is not yet fully understood, but there is accumulating evidence that activation of p53 is mainly achieved by posttranslational modifications in the

N- and C-terminal regions of the protein (reviewed in Giaccia and Kastan, 1998).

The N-terminal region of p53 contains the transactivation domain that mediates the interaction with the basal transcription machinery (Lu and Levine, 1995; Thut *et al.*, 1995), and is also required for p53 degradation (Prives, 1998). It has been demonstrated that this region binds to the proto-oncoprotein Mdm2 (Kussie *et al.*, 1996), resulting in transcriptional silencing (Momand *et al.*, 1992), and, as shown more recently, in ubiquitin/proteasome-dependent degradation of p53 (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). Furthermore, there is evidence to suggest that the phosphorylation status of the N-terminus is involved in p53 stability regulation (Shieh *et al.*, 1997; Khanna *et al.*, 1998; Unger *et al.*, 1999).

Stabilization of the protein does not appear to be the only event required for p53 activation since, at least in some cases, p53-dependent transcription can be stimulated without increase in the protein level (Hupp *et al.*, 1995). The C-terminus seems to play a crucial role in controlling the transcriptional properties of p53. Posttranslational modifications in this domain have been shown to mediate the conversion of the protein from an inert to an active form capable of sequence-specific DNA binding (Hupp and Lane, 1994). Such modifications can be induced by DNA damage and include not only phosphorylation and dephosphorylation (Waterman *et al.*, 1998) but also acetylation and glycosylation (Shaw *et al.*, 1996; Gu and Roeder, 1997). However, the C-terminus may also be involved in modulating the stability of the protein, since deletion of the last 30 amino acids results in a p53 molecule that is still bound by Mdm2 but is refractory to Mdm2-mediated degradation (Kubbutat *et al.*, 1998).

A number of ubiquitin-related proteins have recently been identified (Hodges *et al.*, 1998), including the small ubiquitin-related modifier SUMO-1, which has been reported to be covalently linked to proteins such as RanGAP1, IκBα, Sp100 and PML (Mahajan *et al.*, 1997; Sternsdorf *et al.*, 1997; Desterro *et al.*, 1998). Although the sequence similarity between ubiquitin and SUMO-1 is low (Saitoh *et al.*, 1997), the general mechanism of conjugation is very similar but utilizes different enzymes (Hodges *et al.*, 1998). In contrast to ubiquitination, however, 'sumolation' of a protein does not appear to target it for rapid degradation but rather affects the ability of the modified protein to interact with other cellular factors. For example, covalent modification of RanGAP1 by SUMO-1 affects its subcellular localization and contributes to nuclear pore complex formation (Mahajan *et al.*, 1997; Matunis *et al.*, 1998). In the case of IκBα (Baldwin, 1996), a well established substrate of the ubiquitin/proteasome system, it has been reported that SUMO-1 competes for the lysine residue involved in ubiquitin coupling, providing a possible model for regulation of IκBα degradation

(Desterro *et al.*, 1998). PML and Sp100 are important SUMO-1 conjugated components of the so-called nuclear dots or nuclear bodies (Boddy *et al.*, 1996; Sternsdorf *et al.*, 1997), which are targeted for destruction by immediate early proteins of different DNA viruses at an early stage of infection (Chelbi-Alix and de The, 1999; Muller and Dejean, 1999), suggesting a crucial role for these subnuclear structures in the viral life cycle and more generally in cell proliferation control.

Here we demonstrate that SUMO-1 is conjugated to p53 *in vitro* and *in vivo* and that this covalent modification takes place at the C-terminus of p53 involving a specific lysine residue at position 386. Moreover, we provide evidence that sumolation enhances the transactivation ability of p53. Thus, covalent linkage to SUMO-1 represents a potential novel mechanism to regulate p53 activity.

Results

Isolation of SUMO-1 as a candidate p53 interacting protein

To isolate proteins that interact with p53, the yeast two-hybrid system (Gyuris *et al.*, 1993) was employed using human wt p53 (LexAp53wtΔ74) as a bait. Screening of ~3 million colonies of a fetal brain cDNA library resulted in the isolation of 24 individual clones showing strong and specific interaction with the bait.

Sequence analysis of one of the more representative (20%) clones revealed that it encodes the ubiquitin-related protein SUMO-1 (Boddy *et al.*, 1996; Mahajan *et al.*, 1997). Similar to ubiquitin, SUMO-1 is found in two forms in cells, covalently attached to other proteins and as a free molecule. The enzymes involved in SUMO-1 conjugation have recently been identified. In humans these are Sua1 and hUba2, which form a dimer and, in analogy to the ubiquitin-conjugation system, represent the SUMO-activating enzyme E1 (Desterro *et al.*, 1999; Okuma *et al.*, 1999) and hUbc9, which represents the SUMO-conjugating enzyme E2 (Lee *et al.*, 1998; Schwarz *et al.*, 1998). Interestingly, hUbc9 has previously been reported to interact with p53 in yeast (Shen *et al.*, 1996) and, indeed, we also isolated several clones corresponding to this enzyme.

To further characterize the interaction of p53 with SUMO-1 and hUbc9 in yeast, we used a tumor-derived mutant of p53 (LexAp53H175Δ74) that is conformationally distorted and, as a consequence, has lost the sequence-specific DNA binding properties of wt p53. This revealed that a wt-like conformation is not required for p53 to interact with hUbc9 and SUMO-1. Next, we constructed different LexAp53 deletion mutants to map the region of p53 that is required for the binding. As represented schematically in Figure 1, the interaction with both proteins requires the C-terminal 55 amino acids of p53.

SUMO-1 is conjugated to p53 in vitro

Since other known substrates for SUMO-1 conjugation have been reported to interact with both SUMO-1 and hUbc9 in the yeast two-hybrid system (Boddy *et al.*, 1996; Desterro *et al.*, 1998), we investigated whether p53 may also be a substrate of the SUMO-1 modification pathway. p53 was generated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and incubated in the presence or absence of mouse Ubc9 (mUbc9) (identical to human Ubc9 at the amino acid sequence level), a partially purified protein fraction containing the SUMO-activating enzyme E1, and a GST–SUMO-1 fusion protein (Schwarz *et al.*, 1998). Under the reaction conditions used, a slower migrating form of p53 was observed (Figure 2A, left panel). Since the appearance of this form was dependent on the presence of E1, mUbc9 and GST–SUMO-1, it can be concluded that this form represents p53 molecules modified by the covalent attachment of one moiety of SUMO-1, as judged by its molecular weight. Furthermore, the conjugation of SUMO-1 appeared to be specific for p53 in that HHR23a, a protein that is not related to p53 (Masutani *et al.*, 1994), was not modified under the same assay conditions (Figure 2A, right panel).

The lysine residue at position 386 of p53 is required for SUMO-1 modification

Based on the results obtained in the yeast two-hybrid system, it seemed possible that the C-terminal 55 amino acids of p53 contain all the information that is necessary for p53 to be recognized by the SUMO-1 conjugation system. Indeed, N-terminal deletion mutants of p53 (deletion of the N-terminal 43 amino acids and 293 amino acids, respectively) served as substrates for SUMO-1 conjugation with an efficiency similar to wt p53, while deletion of the C-terminal 30 amino acids resulted in a protein that was not modified by SUMO-1 (data not shown). It is also worth noting that, similar to the results obtained in yeast, a wt-like conformation is not required for the covalent attachment of SUMO-1 to p53 in this *in vitro* system.

To map the lysine residues of p53 that serve as potential attachment sites for SUMO-1, we utilized additional C-terminal deletion mutants of p53. This revealed that removal of the C-terminal 10 amino acids is sufficient to render p53 incompetent for conjugation (data not shown) indicating that a lysine residue within this region, or close to it, may serve as the major attachment site for SUMO-1. The most C-terminal lysine residues of p53 are located at position 381, 382 and 386 (Figure 2B). Therefore, p53 mutants were constructed in which these three residues were changed to arginine in various combinations, and tested for their ability to serve as a substrate for SUMO-1 conjugation. As shown in Figure 2B, mutation of lysine residue 386 results in a protein that cannot be modified by SUMO-1 *in vitro*. In contrast, mutation of Lys 381 and 382, which have been shown to be preferential sites for acetylation (Gu and Roeder, 1997), did not affect the ability of the respective p53 mutant to be modified.

Taken together, our results show that p53 is a substrate for SUMO-1 conjugation *in vitro*. Furthermore, the lysine at position 386 of p53 is required for this modification indicating that this residue serves as the major attachment site for SUMO-1.

p53 is covalently modified by SUMO-1 in vivo

To obtain evidence that p53 is also modified by SUMO-1 in human cells, we performed a Western blot analysis on total lysates from 293 cells. As shown in Figure 3A, a slower migrating p53 form was recognized by the anti-p53 monoclonal antibody DO-1. To determine whether
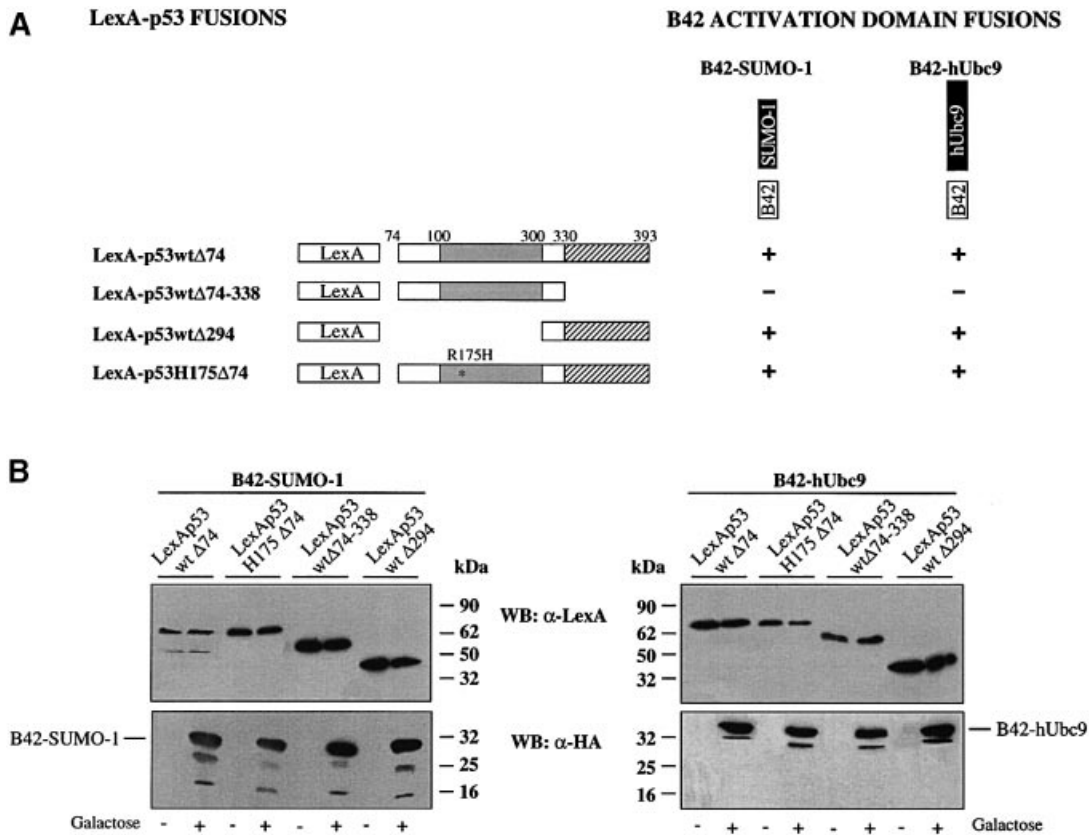
Fig. 1. SUMO-1 and hUbc9 interact with p53 in yeast. (**A**) The different LexA-p53 fusions employed are represented on the left side. p53 domains are indicated: (▨) DNA binding domain; (▧) oligomerization and unspecific DNA binding domains. '+' indicates positive interaction, as judged by β-galactosidase activity and ability to grow in the absence of leucine; '–' indicates no detectable interaction. (**B**) Lysates from cells grown in the absence (–) or in the presence (+) of galactose were analyzed by Western blotting to confirm the expression of the bait (anti-LexA, upper panels) and the fish (anti-HA, lower panels) proteins in the strains used for the interaction assays. The bands corresponding to B42–SUMO-1 and B42–hUbc9 as well as the running position of molecular weight markers are indicated.

this form represents p53 molecules conjugated to SUMO-1, lysates from the same cell line were first immunoprecipitated with a polyclonal antibody against p53 or with pre-immune serum as a negative control. Subsequently, the immunocomplexes were analyzed by Western blotting with an anti-SUMO-1 monoclonal antibody. This revealed that a protein of the expected molecular weight (~70 kDa) was specifically recognized in the anti-p53 immunoprecipitate (Figure 3B, left panel). Finally, the same membrane was stripped and reprobed with the DO-1 antibody to demonstrate that the SUMO-1 crossreactive protein was indeed a modified p53 form (Figure 3B, right panel).

The apparent molecular weight of the immunoprecipitated protein is consistent with the addition of a single SUMO-1 molecule to one p53 molecule. This finding is in agreement with the *in vitro* data presented above, as well as with previous observations that SUMO-1 modification usually takes place on specific single acceptor sites (Desterro *et al*., 1998; Mahajan *et al*., 1998) and that, unlike ubiquitin, poly-SUMO-1 chains are not, or are only very inefficiently, formed (Hodges *et al*., 1998).

SUMO-1 conjugation to p53 in vivo requires lysine 386

To demonstrate that the p53 lysine residue at position 386 is also essential for *in vivo* SUMO-1 conjugation, we generated constructs encoding tagged SUMO-1 proteins by cloning the entire SUMO-1 ORF fused to either the

green fluorescent protein (GFP–SUMO-1) or the HA epitope (HA–SUMO-1). The ability of these fusion proteins to be efficiently conjugated to cellular proteins was then tested in transient transfection experiments followed by Western blot analysis using antibodies against SUMO-1, the HA-tag or the GFP-tag of the respective fusion protein. Consistent with previous reports (Schwarz *et al*., 1998), a high molecular weight smear was observed (data not shown), which indicates that several cellular proteins were covalently modified by conjugation to the tagged SUMO-1. Moreover, both fusion proteins were demonstrated to colocalize with the previously reported SUMO-1 substrates Sp100 and PML (Sternsdorf *et al*., 1997; not shown).

Having shown that GFP–SUMO-1 can be conjugated to cellular proteins, pGFPSUMO-1 was transiently transfected into the p53-null cell line SaOS-2, together with a vector expressing wt p53 (pcDNA3p53wt) or the K386R mutant (pRcCMVp53K386R). At 36 h after transfection, the cells were lysed in SDS sample buffer and analyzed by Western blotting using the p53-specific antibody DO-1. When wt p53 was coexpressed with GFP–SUMO-1 (Figure 4A, lane 3), a more slowly migrating band was visible and its molecular weight (~100 kDa) was consistent with a form of p53 that is covalently modified by GFP–SUMO-1. In contrast, when the p53 mutant (p53K386R), which is not conjugated *in vitro*, was used, no shifted p53-crossreactive band was observed (Figure 4A, lane 5). Similar results were also obtained in Balb/c(10)1 fibroblasts (not shown).
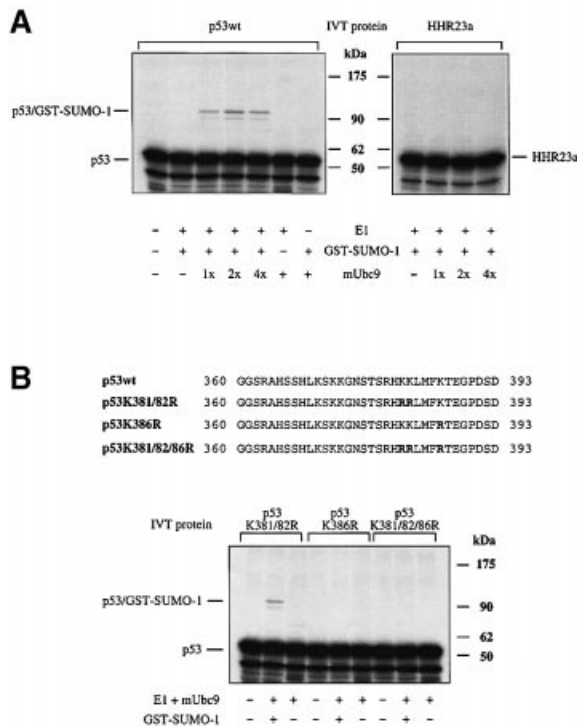
A

B

p53wt   360 GGSRAHSSHLKSKKGNSTSRHKKLMFKTEGPDSD 393
p53K381/82R   360 GGSRAHSSHLKSKKGNSTSRHRRLMFKTEGPDSD 393
p53K386R   360 GGSRAHSSHLKSKKGNSTSRHKKLMFRTEGPDSD 393
p53K381/82/86R   360 GGSRAHSSHLKSKKGNSTSRHRRLMFRTEGPDSD 393

Fig. 2. p53 is a substrate for SUMO-1 conjugation *in vitro*. (**A**) *In vitro* translated ^{35}S-labeled p53 was incubated in the presence (+) or in the absence (–) of the indicated components. After 2 h at 30°C, reactions were stopped and the whole reaction mixtures were separated by SDS–PAGE followed by fluorography. The bands corresponding to free and SUMO-conjugated p53 (left panel) and the running position of molecular weight markers are indicated. As a negative control, the p53 unrelated protein HHR23a was used (right panel). (**B**) *In vitro* translated ^{35}S-labeled p53 mutants, with lysine to arginine substitutions at the indicated positions (p53K381/82R, p53K386R, p53K381/82/86R) were tested for SUMO-1 conjugation *in vitro* as described in (A). The amino acid sequence of the C-terminal region of the various mutants is shown above.
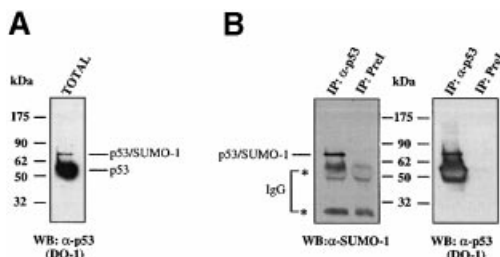

Fig. 3. Endogenous p53 is modified by SUMO-1 *in vivo*. (**A**) 293 cells were directly lysed in SDS-containing sample buffer and samples were analyzed by SDS–PAGE followed by Western blotting using the anti-p53 monoclonal antibody DO-1. The positions of free and putative SUMO-1 conjugated p53 are indicated. Molecular weight markers are shown on the left. (**B**) Lysates from 293 cells were immuno-precipitated with a polyclonal antiserum raised against human p53 (α-p53) or with pre-immune serum (PreI) as a negative control. Subsequently, immunoprecipitates were analyzed by Western blotting with an anti-SUMO-1 monoclonal antibody (left panel). The same membrane was then stripped and reprobed with DO-1 (right panel). Running position of molecular weight markers and of SUMO-1 conjugated p53 are indicated.

To demonstrate directly that the higher band observed in the above experiment corresponds to p53 covalently linked to GFP–SUMO-1, SaOS-2 cells were transiently transfected with wt p53 together with GFP–SUMO-1 or with GFP. Cell lysates were immunoprecipitated with an
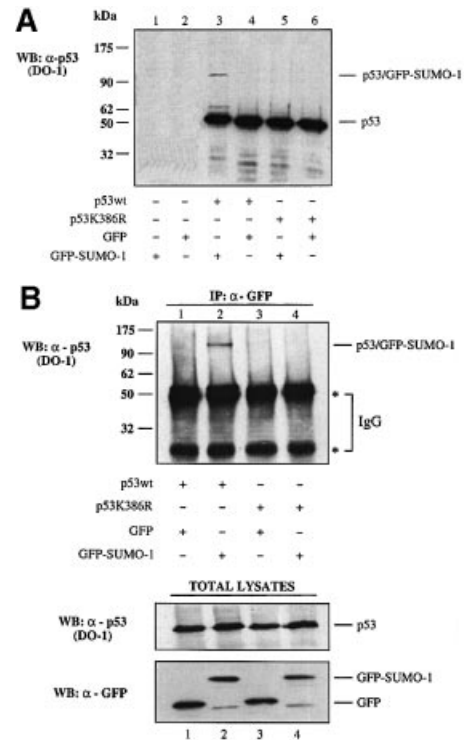

Fig. 4. Lysine 386 is required for SUMO-1 conjugation to p53 *in vivo*. (**A**) SaOS-2 cells were seeded in 6 cm diameter Petri dishes and transfected with 1 µg of p53 expression plasmids and 2 µg of pGFPSUMO-1 or empty pGFP. Total extracts were prepared by direct lysis in SDS sample buffer, resolved by SDS–PAGE and analyzed by Western blotting using the anti-p53-specific antibody DO-1. Positions of molecular weight markers, free p53 and putative p53/GFP–SUMO-1 conjugates are indicated. (**B**) Lysates from SaOS-2 cells transfected with 4 µg of p53 expression plasmids and 10 µg of pGFP or pGFP-SUMO-1 as indicated were immunoprecipitated with an anti-GFP polyclonal antibody and immunoprecipitates were subjected to Western blot analysis using the DO-1 antibody (upper panel). An aliquot of each lysate was checked for the expression of the transfected plasmids by staining with DO-1 (middle panel) or anti-GFP (lower panel) antibodies. Running positions of molecular weight markers and of the various proteins are indicated.

anti-GFP polyclonal antibody and then blotted with DO-1. As shown in Figure 4B (upper panel), a 100 kDa p53-reactive band was specifically immunoprecipitated by the anti-GFP antibody but only when GFP–SUMO-1 was coexpressed (Figure 4B, lane 2). In contrast, no GFP–SUMO-1-linked p53 was detected when the conjugation-deficient mutant K386R was employed (Figure 4B, lane 4).

Taken together, these results demonstrate that p53 can be covalently modified by SUMO-1 *in vitro* and *in vivo*. Furthermore, the Lys386 residue identified in *in vitro* experiments is also required for SUMO-1 modification *in vivo*.

The C-terminal region of p53 is sufficient to be targeted by SUMO-1 conjugation in vivo

The results obtained in the yeast two-hybrid system and the *in vitro* sumolation assay indicated that the C-terminus of p53 may be sufficient for recognition as a substrate for SUMO-1 modification. To test if this domain is also sufficient in mediating the conjugation *in vivo*, we ectopically expressed a construct encoding the last 100 amino acids of p53, in fusion with the HA epitope (pcDNA3HAp53Ct), together with HA–SUMO-1. The
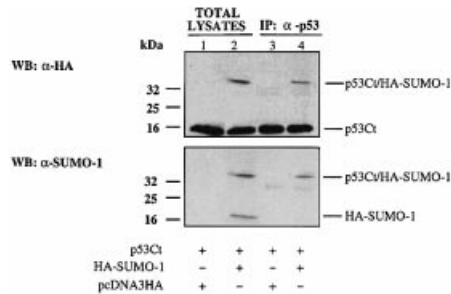
Fig. 5. The C-terminal region of p53 is sufficient for SUMO-1 conjugation. Total extracts (lanes 1 and 2) and anti-p53 immunoprecipitates (lanes 3 and 4) from U2OS cells transfected with 4 µg of pcDNA3HAp53Ct (encoding amino acids from 294 to 393) together with 10 µg of empty pcDNA3HA vector or pcDNA3HASUMO-1 were analyzed by Western blotting with anti-HA (upper panel) or anti-SUMO-1 (lower panel) specific antibodies. Running positions of molecular weight markers and the positions of free p53Ct and HA–SUMO-1 as well as of the conjugated proteins are indicated.

p53Ct protein contains p53 nuclear localization signals and is efficiently localized to the nucleus (not shown). Cell lysates were immunoprecipitated with an anti-p53 polyclonal antibody and subsequently analyzed by Western blotting with anti-HA and anti-SUMO-1 antibodies. As shown in Figure 5, in total lysates (upper panel, lane 2) as well as in immunoprecipitates (upper panel, lane 4) from cells expressing HA–SUMO-1, a slower migrating HA-crossreactive band was present. When the membrane was stripped and reprobed, the same band was recognized by the anti-SUMO-1 specific antibody (Figure 5, lower panel, lanes 2 and 4). No protein was detected with either of the antibodies in pcDNA3HA control transfected cells (Figure 5, lanes 1 and 3). Thus, the C-terminal region of p53 is likely to be sufficient for recognition as a substrate by the SUMO-1 conjugation pathway. However, a role of other domains of the protein in modulating the efficiency of the modification cannot be excluded. Finally, it should be noted that a wt-like conformation of the full-length p53 does not appear to be required for *in vivo* sumolation, since the tumor-derived mutant p53R175H was also linked to GFP–SUMO-1 in cells (data not shown).

SUMO-1 modification enhances p53-dependent transactivation

Recent results point to the important role of the C-terminus in p53 degradation (Kubbutat *et al.*, 1998) and functional activation (Hupp and Lane, 1994). Furthermore, posttranslational modification of the C-terminal 30 amino acids by phosphorylation (Hupp *et al.*, 1992) or acetylation (Gu and Roeder, 1997) has been demonstrated to modulate the ability of p53 to bind DNA and to exert its function as a transcriptional activator. Therefore, it was of interest to determine whether the conjugation of SUMO-1 to the C-terminal region of p53 affects its transactivation capacity. To test this possibility, a luciferase reporter construct containing the p21 promoter (p21-Luc; el-Deiry *et al.*, 1993) was transfected into U2OS cells, which contain endogenous wt p53, together with the empty pcDNA3HA vector or with increasing amounts of pcDNA3HASUMO-1. As shown in Figure 6A, HA–SUMO-1 overexpression enhanced luciferase activity from the p21 reporter up to 3-fold and this increase correlated

with the amount of overexpressed protein, as detected by Western blot analysis (Figure 6A, lower panel). Reporter activity from a plasmid lacking p53 binding sites was not affected by HA–SUMO-1 overexpression (data not shown). Similar results were obtained with GFP–SUMO-1 (not shown). This indicates that sumolation of p53 increases its transcriptional activity.

To obtain further evidence that the observed increase of p21-Luc activity was due to SUMO-1 modification of p53, p21-Luc was transfected into p53-null Balb/c(10)1 fibroblasts together with vectors expressing either wt p53 or the conjugation-deficient mutant p53K386R. The basal levels of luciferase activity obtained with the two proteins were comparable (Figure 6B, bars 1 and 3). However, when HA–SUMO-1 was coexpressed, a significant increase in the activation of the reporter was observed only in cells expressing wt p53 (Figure 6B, bars 1 and 2), with the activity of the mutant protein being unaffected by HA–SUMO-1 (Figure 6B, bars 3 and 4). As a control, the basal activity of the p21-Luc reporter in the absence of p53 (Figure 6B, bars 5 and 6) did not significantly change following HA–SUMO-1 overexpression.

To exclude the possibility that the different activities observed for wt p53 and the K386R mutant were not due to variations in expression levels, an aliquot of the lysates was subjected to Western blot analysis using the monoclonal antibody DO-1. As shown in Figure 6B (lower panel), p53 expression levels were comparable under the conditions used.

Since Ubc9 is the E2 enzyme that mediates SUMO-1 conjugation, we tested the possibility that hUbc9 overexpression enhances the transactivation capacity of p53 by increasing the fraction of p53 modified by endogenous SUMO-1. Therefore, luciferase assays were performed with lysates from U2OS cells transfected with p21-Luc and increasing amounts of pcDNA3HAhUbc9. As for HA–SUMO-1, HA–hUbc9 overexpression resulted in enhanced reporter activity (Figure 6C). Also in this case, the observed effect was most likely directly dependent on the presence of conjugation-competent p53, since no increase in luciferase activity was obtained when HA–hUbc9 was coexpressed in Balb/c(10)1 fibroblasts together with p53K386R (Figure 6C).

SUMO-1 and hUbc9 act in a cooperative way in modifying p53

The results presented above suggested that both hUbc9 and SUMO-1 are rate-limiting factors in the conjugation of SUMO-1 to p53 and, thus, coexpression of SUMO-1 and hUbc9 may have a cooperative effect on the transactivation activity of p53. To test this hypothesis, U2OS cells were transfected with GFP–SUMO-1 and HA–hUbc9, either separately or together and p21-Luc luciferase activity was determined. As shown in Figure 7A, when both proteins were expressed (bar 4) the increase in the p21-Luc reporter activity was 2-fold higher than in cells expressing only GFP–SUMO-1 (bar 2) or HA–hUbc9 (bar 3). In all the samples, expression of the different fusion proteins was controlled by Western blotting (Figure 7B, central panels). Furthermore, to test whether conjugation of SUMO-1 to p53 also resulted in enhanced expression of the endogenous p21 protein, the lysates used for the luciferase assay were analyzed by Western blotting using an anti-p21 polyclonal
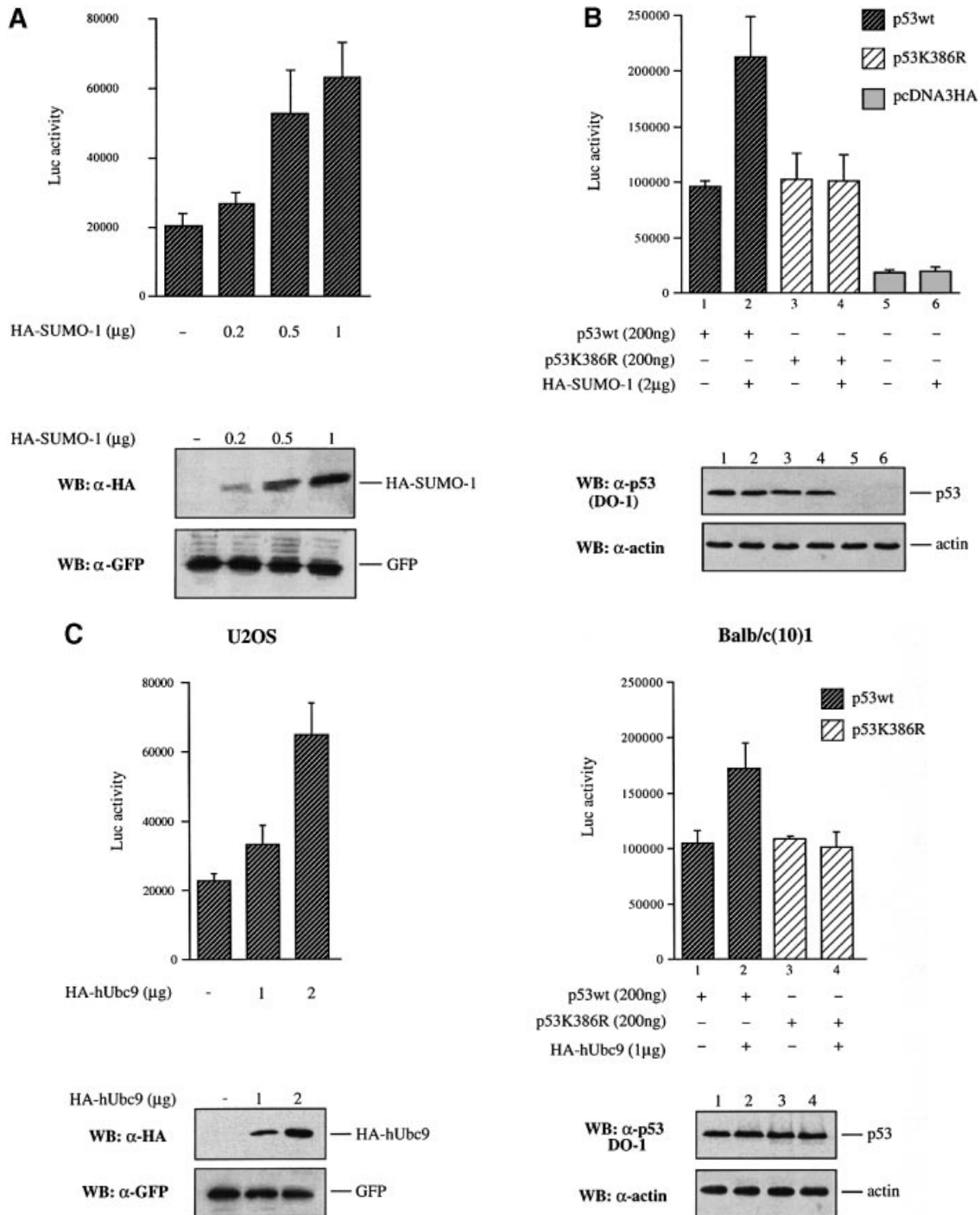
Fig. 6. SUMO-1 conjugation enhances p53 transcriptional activity. (**A**) Luciferase assays were performed on lysates from U2OS cells transfected with a p21-Luc reporter plasmid together with empty vector or increasing amounts of pcDNA3HASUMO-1, as indicated. In addition, 100 ng of pGFPC1 were cotransfected to monitor the efficiency of transfection. An aliquot of the lysates was analyzed by Western blotting with anti-HA antibody to confirm the expression of the transfected HA–SUMO-1 and with anti-GFP antibody to evaluate the efficiency of transfection (lower panels). (**B**) Balb/c(10)1 fibroblasts were transfected with the p21-Luc reporter, pcDNA3p53wt (▨) or pRcCMVp53K386R (▧) or empty pcDNA3HA vector (▦), either with or without HA–SUMO-1. Luciferase assay was performed as in (A). An aliquot of each lysate was analyzed by Western blotting using the DO-1 antibody to demonstrate comparable levels of expression of p53 in all the samples. The same membrane was subsequently probed with an anti-actin antibody to estimate the total amount of protein loaded in each lane (lower panels). (**C**) U2OS (left part) or Balb/c(10)1 (right part) cells were transfected with the indicated plasmids and lysates were subjected to luciferase assay. An aliquot of the lysates was analyzed by Western blotting as described in (A) (left part) and (B) (right part), respectively. In all cases, graphs represent the mean of at least three independent experiments. Standard deviations are indicated.

antibody. This revealed that the expression of p21 was increased in cells expressing either SUMO-1 or hUbc9 or both (Figure 7B, upper panel).

Finally, to obtain evidence that the observed increase

in the transactivation ability of p53 was indeed linked to an increase in the amount of SUMO-conjugated p53, SaOS-2 cells were transfected with wt p53 and increasing amounts of GFP–SUMO-1 in the presence or absence of
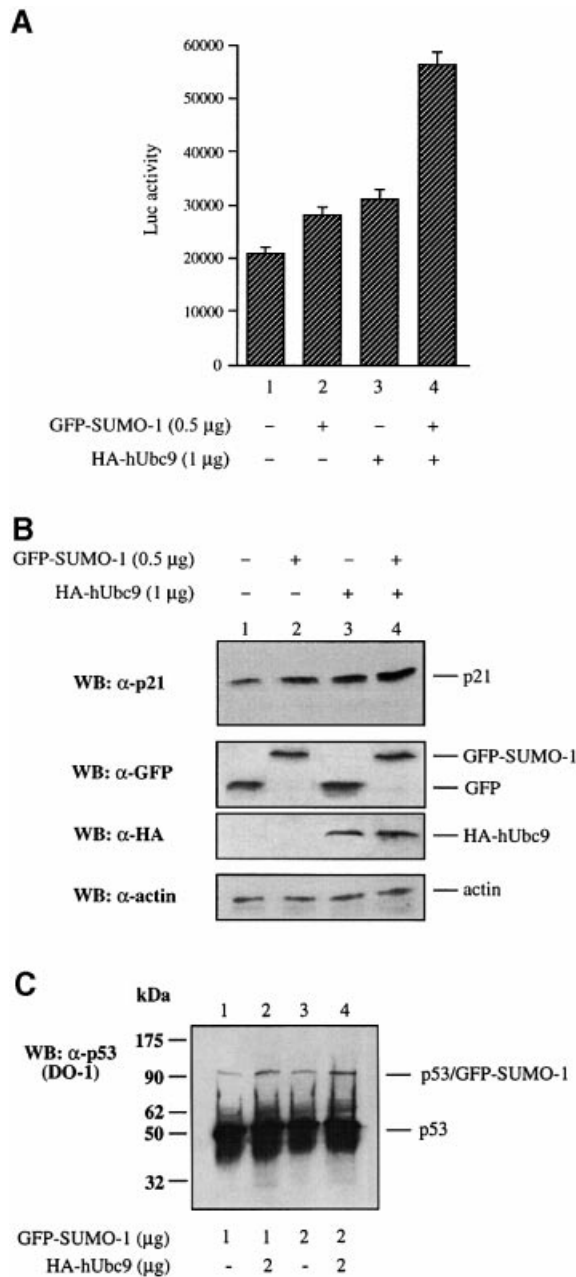
Fig. 7. SUMO-1 and hUbc9 have a cooperative effect on p53 sumolation and transcriptional activation. (**A**) Luciferase assays were performed on extracts from U2OS cells transfected with p21-Luc and with either pGFPSUMO-1 or pcDNA3HAhUbc9 or both plasmids, as indicated. The graph represents the mean of three independent experiments. Standard deviations are indicated. (**B**) An aliquot of the lysates used for the luciferase assay was subjected to Western blot analysis with an anti-p21 polyclonal antibody (upper panel) and with anti-HA or anti-GFP antibodies to check the expression of the transfected proteins (central panels). Comparable amounts of proteins were loaded in each lane, as estimated by probing the same membrane with anti-actin antibody (lower panel). (**C**) SaOS-2 cells were transfected with 1 µg of pcDNA3p53wt together with the indicated amounts of plasmid encoding GFP–SUMO-1 and HA–hUbc9. Total lysates were then analyzed by Western blotting with the anti-p53 monoclonal antibody DO-1. A shorter exposure of the membrane revealed similar amounts of the unmodified form of p53 in all of the lanes (not shown). Positions of free and GFP–SUMO-1-conjugated p53 and molecular weight markers are shown.

overexpressed HA–hUbc9 and total lysates were subjected to Western blot analysis with the p53-specific antibody DO-1 (Figure 7C). As expected, in the absence of exogenous hUbc9, elevating the amount of transfected GFP–SUMO-1 resulted in a simultaneous increase in the level of sumolated p53 (Figure 7C, compare lanes 1 and 3). Coexpression of HA–hUbc9 resulted in a further increase of the modified form of p53 (lanes 2 and 4), demonstrating that indeed both SUMO-1 and hUbc9 are limiting factors in the conjugation process.

Discussion

The growth-suppressive properties of p53 appear to be regulated by at least two general mechanisms that are not mutually exclusive. Under normal growth conditions, p53 is expressed at low levels, which are at least in part due to the short half-life of the protein. In response to a variety of stress signals, however, p53 has a significantly extended half-life resulting in its accumulation. Furthermore, p53 is subject to posttranslational changes that may affect either its overall structure, and thus its biochemical properties, or its turnover rate, or both. In this study we report that p53 can be covalently modified by conjugation to the ubiquitin-like protein SUMO-1 on a single lysine residue (K386) that is localized in the extreme C-terminus of the protein and that is highly conserved during evolution. Furthermore, this posttranslational modification enhances the transactivation activity of p53. Thus, conjugation of SUMO-1 to p53 provides a previously undescribed modification that probably contributes to control the growth-suppressive properties of p53.

Similar to other known substrates of the SUMO-1 conjugation system, modification of p53 by SUMO-1 requires the activity of the SUMO-activating enzyme E1 and the SUMO-conjugating enzyme hUbc9. Accordingly, we have shown that p53 transcriptional activity is enhanced by SUMO-1 or hUbc9 overexpression, indicating that both factors are rate-limiting in the conjugation process. This hypothesis is supported by the observation that overexpression of SUMO-1 together with hUbc9 increases the amount of sumolated p53. In this context, it should be noted that only a small percentage of the total p53 appears to be modified by SUMO-1 *in vitro* and *in vivo*. This observation may be explained by the possibility that an additional factor(s) is required to target hUbc9 to p53 for SUMO-1 conjugation. Similarly, it is possible that only a subfraction of p53 is recognized as a substrate by the SUMO conjugation system since, for example, recognition may require prior modification of p53 by phosphorylation or acetylation. The wt conformation of p53, however, is not required, since both wt p53 and the tumor-derived H175 mutant are sumolated with similar efficiencies (not shown). Alternatively, SUMO-conjugated p53 may be subjected to the action of SUMO-specific proteases that revert SUMO-conjugated p53 to its non-modified form. Interestingly, a SUMO-specific protease has recently been described that is required for cell cycle progression in yeast (Li and Hochstrasser, 1999). Thus, it is tempting to speculate that conjugation of SUMO-1 to p53 may directly affect its growth-suppressive properties. Further studies will be required to address this issue.

How is p53 activated by conjugation to SUMO-1?

Based on previous results, several mechanisms can be envisioned, including allosteric regulation, interference with ubiquitin/proteasome-mediated degradation, and changes in subcellular distribution. With respect to allosteric regulation, it has been proposed that the C-terminus of p53 interacts with the core domain, thereby keeping the protein in an inactive form. Indeed, removal of the C-terminal 30 amino acids of p53 or modification of this region by phosphorylation or acetylation, activate the sequence-specific DNA binding activity, probably by changing the overall conformation of the protein (Hupp and Lane, 1994; Gu and Roeder, 1997). Since sumolation occurs within the C-terminal 30 amino acids of p53, a similar mechanism may account for the observed increase in the transactivation activity of p53 upon SUMO-1 conjugation. Alternatively, several cellular proteins have been shown to interact with the C-terminus of p53 and, thus, the presence of SUMO-1 on Lys386 may alter the capacity of p53 to interact with these proteins. Along these lines, it will be interesting to determine if sumolation of p53 affects the phosphorylation and/or acetylation status of its C-terminal region.

It has recently been demonstrated that binding of Mdm2 induces the rapid degradation of p53 via the ubiquitin/proteasome system (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Interestingly, a deletion mutant of p53 in which the C-terminal 30 amino acids were removed was still bound by Mdm2 but was not targeted for degradation (Kubbutat *et al.*, 1998). Thus, similarly to the allosteric model discussed above, sumolation may prevent Mdm2-targeted degradation of p53, which in turn, would be expected to result in enhanced transactivation activity. Alternatively, in analogy to IκBα degradation (Desterro *et al.*, 1998), sumolation of p53 may inhibit its degradation by competing for the same lysine residue that is required for p53 ubiquitination or by interfering with conjugation of ubiquitin molecules to neighboring sites. Although these models may prove to be correct for endogenous p53, in our hands, transiently overexpressed p53 has in general a significantly increased half-life (A.Hengstermann and M.Scheffner, unpublished data) and consequently we did not observe an increase in p53 levels upon coexpression of SUMO-1 and/or hUbc9. Therefore, interference with p53 degradation may not significantly contribute to the observed increase in p53 transactivation activity in the transient transfection assays presented above (see Figure 6B).

Another issue to be addressed is the role of the sumolation pathway in the subcellular distribution of p53. Interestingly, it has been reported that leptomycin B treatment or coexpression of p53 together with Mdm2 and ARF can induce the localization of p53 into discrete subnuclear structures, reminiscent of the PML/Sp100 nuclear bodies (Lain *et al.*, 1999; Zhang and Xiong, 1999). Our preliminary results suggest that, in the presence of SUMO-1 and hUbc9, p53 indeed relocalizes to similar structures (V.Fogal and G.Del Sal, unpublished data). Therefore, an attractive but purely speculative hypothesis is that, within these structures, p53 interacts with other resident factors (e.g. p300/CBP) to perform specific transcription functions. However, further work is required to clarify this point.

Although the exact mechanism remains to be deter-mined, modification of p53 by conjugation to SUMO-1 clearly can affect the activity of p53. Thus, modulation of this pathway, e.g. by enhancing the sumolation of p53 or by inhibiting the removal of SUMO-1 by specific proteases (Li and Hochstrasser, 1999), may provide a novel platform for developing alternative strategies to modulate p53 response.

Materials and methods

Cell lines
All the cell lines used were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). U2OS and SaOS-2 cells are human osteosarcoma cell lines, wt and null for p53, respectively. The Balb/c(10)1 fibroblast cell line is a murine cell line that does not contain endogenous p53. 293 is a human embryonic kidney cell line.

Plasmids
To generate the LexA-fusion constructs, human wt p53 and p53H175 cDNAs were PCR-amplified and cloned in-frame into pLexA202 (Gyuris *et al.*, 1993). pcDNA3p53wt contains the full-length human wt p53 cDNA cloned by *Eco*RI into pcDNA3 (Invitrogen). cDNAs encoding the p53 mutants K386R, K381/82R and K381/82/86R were generated by PCR-directed mutagenesis and the respective cDNAs were cloned by *Hin*dIII and *Xba*I restriction into pRcCMV (Invitrogen). To construct pcDNA3HAp53Ct, a cDNA fragment of human p53 encoding amino acids 294–393 was PCR-amplified and cloned downstream of the HA epitope into pcDNA3 (pcDNA3HA). To generate the different SUMO-1 fusions, a cDNA encoding SUMO-1 was PCR-amplified and cloned by *Bam*HI–*Xho*I into pcDNA3HA or by *Bam*HI–*Xba*I into pGFPC1 (Clontech). hUbc9 was sublcloned from pJG4-5 into pcDNA3HA by *Eco*RI–*Xho*I. All PCR-amplified products were fully sequenced to exclude the possibility of second site mutations. The p53-reporter plasmid employed for luciferase assays, p21-Luc, has been described previously (el-Deiry *et al.*, 1993).

Yeast two-hybrid screen
p53wtΔ74 fused to the LexA DNA-binding domain was introduced into the EGY48/pSH1834 yeast strain (Gyuris *et al.*, 1993). The resulting strain was then transformed with a human fetal brain cDNA library cloned into the pJG4-5 plasmid. The screening was performed as described previously (Gyuris *et al.*, 1993). For Western blot analysis, cells grown in medium containing either glucose or galactose and raffinose (to induce the expression of the 'fish' protein) were subjected to mechanical lysis with glass beads in SDS-containing sample buffer. Expression of the bait proteins was assessed using an anti-LexA polyclonal antibody (Invitrogen). Expression of the fish proteins was controlled using the monoclonal antibody 12CA5 (Roche Molecular Biochemicals) against the HA tag present in the pJG4-5 vector, after the B42 activation domain.

In vitro conjugation assay
The various forms of p53 and the p53-unrelated protein HHR23a (Masutani *et al.*, 1994) were generated in the TNT rabbit reticulocyte lysate system in the presence of [^{35}S]methionine according to the manufacturer's instructions (Promega). Murine Ubc9 and GST–SUMO-1 were expressed in *Escherichia coli* BL21(DE3) and *E.coli* DH5α, respectively, as described (Schwarz *et al.*, 1998). Crude bacterial extracts were used as a source of mUbc9. GST–SUMO-1 was purified by affinity chromatography using glutathione–Sepharose (Amersham Pharmacia, Biotech). As a source of SUMO-activating enzyme activity, protein extracts were prepared from confluent NIH 3T3 cells and fractionated by anion exchange chromatography on a 1 ml Mono Q column as described previously (Schwarz *et al.*, 1998).

SUMO conjugation assays were performed in reaction mixtures containing 10 μg of the Mono Q fraction of the NIH 3T3 cell extracts, 100–300 ng of mUbc9, 4 μg GST–SUMO-1, 1 μl of radiolabeled p53 or HHR23a in 25 mM Tris–HCl pH 7.5, 2 mM ATP, 4 mM MgCl$_2$, 1 mM dithiothreitol. After 2 h at 30°C, reactions were terminated by boiling the mixtures in SDS-containing buffer. Reaction mixtures were separated on 10% SDS–polyacrylamide gels and radioactively labeled bands were visualized by fluorography.

Transfections and luciferase assays

Transfections were performed by the standard calcium phosphate precipitation method. Cells were seeded 8 h before transfection and processed for a further 24 h after removal of the precipitate. For luciferase assay, 6 cm Petri dishes were transfected with 500 ng of the reporter construct and the indicated amount of other plasmids. The assay was performed with the luciferase kit from Promega. Luciferase activity was determined in a Turner Design luminometer (Promega). The values obtained were normalized for protein concentration in each sample, as determined by a colorimetric assay (Bio-Rad Protein Assay).

Immunoprecipitation and Western blot analysis

Subconfluent cells seeded on 10 cm diameter Petri dishes were transfected with the expression vectors indicated. At 36 h after transfection, cells were washed with ice-cold phosphate-buffered saline (PBS), then harvested in 1 ml of ice-cold RIPA buffer containing 10 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml each of chymostatin, leupeptin, antipain and pepstatin. Lysis was performed at 4°C for 20 min. Then, the lysates were clarified by centrifugation, precleared with 200 µl of Immunoprecipitin (Gibco-BRL) and finally incubated for 4 h at 4°C with the respective antibodies, prebound to 20 µl of protein A–Sepharose CL-4B (Amersham Pharmacia, Biotech). The beads were then washed three times in 1 ml of ice-cold lysis buffer and the bound proteins were solubilized by addition of 20 µl of SDS-containing sample buffer. In the case of GFP–SUMO-1 immunoprecipitations, 2 µg of anti-GFP polyclonal antibody (Invitrogen) were used, while for p53 immunoprecipitations we used 10 µl of polyclonal antiserum raised against human p53 expressed in bacteria as a GST fusion protein. In the case of p53Ct immunoprecipitation, antibodies were covalently crosslinked to protein A–Sepharose beads. Western blot analysis was performed according to standard procedures using the following primary antibodies: 12CA5 (monoclonal anti-HA, Roche Molecular Biochemicals), DO-1 (monoclonal anti-p53), 21C7 (monoclonal anti-SUMO-1, Zymed), polyclonal anti-GFP (Invitrogen), polyclonal anti-actin (Sigma), polyclonal anti-p21 C19 (Santa Cruz). Bound primary antibodies were visualized by enhanced chemiluminescence (Amersham, Pharmacia, Biotech).

Acknowledgements

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