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SUMO-1 modification activates the transcriptional response of p53

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The p53 tumour suppressor protein is regulated by ubiquitin-mediated proteasomal degradation. In normal cells p53 is constitutively ubiquitylated by the Mdm2 ubiquitin ligase. When the p53 response is activated by stress signals p53 levels rise due to inhibition of this degradative pathway. Here we show that p53 is modified by the small ubiquitin-like protein SUMO-1 at a single site, K386, in the C-terminus of the protein. Modification *in vitro* requires only SUMO-1, the SUMO-1 activating enzyme and ubc9. SUMO-1 and ubiquitin modification do not compete for the same lysine acceptor sites in p53. Overexpression of SUMO-1 activates the transcriptional activity of wild-type p53, but not K386R p53 where the SUMO-1 acceptor site has been mutated. The SUMO-1 modification pathway therefore acts as a potential regulator of the p53 response and may represent a novel target for the development of therapeutically useful modulators of the p53 response.

Keywords: mdm2/p53/SUMO-1/ubiquitin

Introduction

The p53 tumour suppressor plays a significant part in the response of the cell to genotoxic damage. The important role of p53 in maintenance of genome integrity is illustrated by the loss of p53 function in most human tumours (Harris and Hollstein, 1993) and the high rate of tumour development in p53 knockout mice (Armstrong *et al.*, 1995; Donehower, 1996). p53 is a transcription factor that binds to specific sequences in the upstream region of many genes whose protein products regulate cell cycle progression and apoptosis (Ko and Prives, 1996). Thus, cells with damaged DNA do not proliferate and the loss of this protective function allows the uncontrolled growth of cells containing oncogenic mutations (Lane, 1992).

In normal cells p53 is maintained at a low level by rapid turnover of the protein. Exposure of cells to a range of different stress signals, including DNA damage, hypoxia and heat shock, results in a dramatic increase in the half-life of p53 and a consequent increase in the level of the protein (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991; Fritsche *et al.*, 1993). This accumulation of p53 is responsible for the increased transcription of cellular genes, the products of which are associated with cell cycle arrest ($p21^{Waf1Cip1}$) or apoptosis (Bax), and constitute a significant part of the p53 response (Lu and Lane, 1993; Brugarolas *et al.*, 1995; Waldman *et al.*, 1995; McCurrach *et al.*, 1997; Yin *et al.*, 1997). The rapid turnover of the p53 protein is via the ubiquitin–proteasome pathway (Maki *et al.*, 1996) and this is mediated by association with mdm2 (Bottger *et al.*, 1997; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). As transcription of the *mdm2* gene is dependent on p53, this establishes an autoregulatory loop in which p53 induces synthesis of a gene product that targets p53 for destruction (Barak *et al.*, 1993; Wu *et al.*, 1993). Although direct interaction of mdm2 with p53 is thought to inhibit the transcription activity of p53 (Momand *et al.*, 1992; Chen *et al.*, 1993; Oliner *et al.*, 1993), recent experiments indicate that the important activity of mdm2 is to act as an E3 ubiquitin protein ligase that directly transfers ubiquitin onto p53 (Honda *et al.*, 1997). Thus, events that lead to blockage of the interaction between p53 and mdm2 will lead to stabilization of p53 and activation of the p53 response. Phosphorylation of p53 by DNA-dependent protein kinase reduces the affinity of the interaction between p53 and mdm2 (Shieh *et al.*, 1997) and as a consequence phosphorylated p53 is a poor substrate for the ubiquitin ligase activity of mdm2 (Honda and Yasuda, 1999). The $p19^{ARF}$ tumour suppressor interacts with mdm2 and blocks its ability to inhibit p53 activity (Pomerantz *et al.*, 1998). This is a direct consequence of inhibition of the ubiquitin protein ligase activity of mdm2 (Honda and Yasuda, 1999).

Ubiquitin addition is accomplished via a thioester cascade with ubiquitin first being activated by a unique E1 enzyme that utilizes ATP to adenylate the C-terminal glycine of ubiquitin. Release of AMP accompanies the formation of a thioester bond between the C-terminus of ubiquitin and a cysteine residue in the E1 protein. In a transesterification reaction the ubiquitin is transferred from the ubiquitin activating enzyme to an E2 ubiquitin conjugating enzyme, which may in turn transfer the ubiquitin to an E3 ubiquitin protein ligase. In many cases it is this enzyme that recognizes the protein substrate and catalyzes formation of an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of lysine in the target protein. Proteins destined for degradation via the proteasome are coupled to multiple copies of ubiquitin by formation of further isopeptide bonds between additional ubiquitin molecules and lysine residues in the bound ubiquitin (Hershko and Ciechanover, 1992).

Whereas addition of multiple copies of ubiquitin targets proteins for degradation, it is now widely recognized that covalent attachment of other ubiquitin-related molecules

does not result in degradation of the modified protein. Recently a small ubiquitin-like protein variously known as SUMO-1, sentrin, GMP1, UBL1 and PIC1 has been found covalently linked to Ran GTPase activating protein 1 (RanGAP1) and associated with a variety of other proteins (Boddy *et al.*, 1996; Matunis *et al.*, 1996; Shen *et al.*, 1996; Kamitani *et al.*, 1997; Mahajan *et al.*, 1997). Covalent modification of RanGAP1 appears to be necessary for its interaction with the Ran-GTP-binding protein, RanBP2, at the cytoplasmic face of the nuclear pore complex (Mahajan *et al.*, 1997; Saitoh *et al.*, 1997), while SUMO-1 modification of promyelocytic leukaemia gene product (PML) targets the protein to PML oncogenic domains (Sternsdorf *et al.*, 1997; Muller *et al.*, 1998; Duprez *et al.*, 1999). SUMO-1 modification of IκBα takes place on the same residues used for ubiquitylation, thus rendering the modified protein resistant to signal-induced degradation and consequently blocking NF-κB-dependent transcriptional activation (Desterro *et al.*, 1998). SUMO-1 and Smt3p, a yeast homologue of SUMO-1, are conjugated to target proteins by a pathway that is distinct from, but analogous to, ubiquitin conjugation. The SUMO-1 activating enzyme (SAE) catalyzes the ATP-dependent activation of SUMO-1, the first step in the conjugation pathway (Desterro *et al.*, 1999; Gong *et al.*, 1999; Okuma *et al.*, 1999). Furthermore, this enzyme could also transfer activated SUMO-1 to Ubch9, the E2 conjugating enzyme involved in this process (Desterro *et al.*, 1997; Johnson and Blobel, 1997; Saitoh *et al.*, 1998; Schwarz *et al.*, 1998). While the E1 activity for ubiquitin is contained within a single large polypeptide, the E1 activity of SUMO-1 is partitioned between two smaller polypeptides, SAE1 and SAE2. Sequence comparisons between the E1 enzymes indicates that SAE1 is homologous to Aos1p, Ula1p and the N-terminus of the ubiquitin-activating enzymes while SAE2 is homologous to Uba2p, Uba3p and the C-terminus of the ubiquitin-activating enzymes. *In vitro* experiments have demonstrated that SUMO-1 conjugation to IκBα and RanGAP1 can be accomplished in the presence of SEA and Ubc9, indicating that the equivalent of an E3 protein ligase is not required (Desterro *et al.*, 1999; Okuma *et al.*, 1999).

Here we demonstrate that after exposure of cells to UV irradiation p53 is covalently bound to SUMO-1. This modification takes place at a single site, K386, in the C-terminus of p53. SUMO-1 and ubiquitin modification do not compete for the same lysine residue in p53. Overexpression of SUMO-1 activates the transcriptional activity of wild-type p53, but not K386R p53 where the SUMO-1 acceptor site has been mutated. Thus, SUMO-1 modification of p53 represents an additional regulator of p53 stability and may contribute to activation of the p53 response.

Results

p53 is modified by SUMO-1 in vivo

Previous observations indicated that, in the case of IκBα, ubiquitin-mediated proteolysis could be antagonized by modification with the small ubiquitin-like protein SUMO-1 (Desterro *et al.*, 1998). As the p53 response to genotoxic insult is also critically dependent on ubiquitin-mediated proteolysis we investigated the metabolism of p53 in

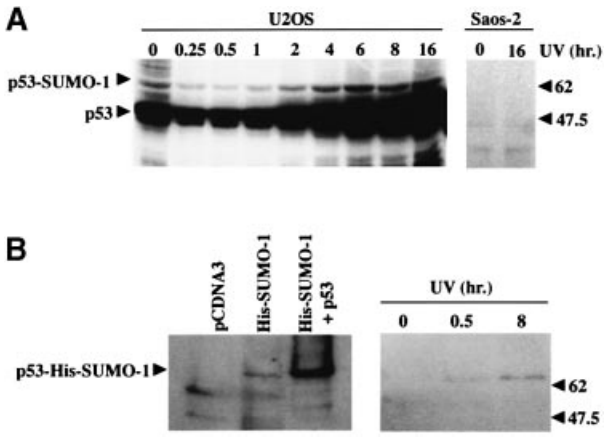

Fig. 1. UV irradiation leads to accumulation of p53 and the SUMO-1 conjugated form of p53 in U20S cells. (**A**) U2OS cells were exposed to 25 J/m^2 of UV irradiation and then collected by direct lysis in SDS buffer at the times indicated. Samples were fractionated by SDS–PAGE and p53 was analysed by Western blotting using a p53-specific monoclonal antibody, DO-1. Soas-2 cells, which lack p53 (right panel), were analysed to demonstrate antibody specificity. (**B**) His$_6$-tagged SUMO-1 is conjugated to p53 *in vivo*. U2OS cells were transfected with pcDNA3, His$_6$-SUMO-1 and p53 as indicated and after 36 h of expression were lysed in buffer containing guanidinium-HCl. Proteins linked to His$_6$-SUMO-1 were purified using Ni^{2+}-NTA-agarose beads (see Materials and methods) and after extensive washing eluted with 200 mM imidazole. Eluted proteins were fractionated by SDS–PAGE and transferred to a nitro-cellulose membrane. His$_6$-SUMO-1 p53 conjugates were detected by Western blotting using anti-p53 antibody (DO-1). Molecular weight markers are shown on the right. Positions of p53 and of SUMO-1 conjugated p53 are indicated by arrowheads. In the right panel His$_6$-SUMO-1 transfected cells were exposed to UV irradiation and analysed as indicated above.

U2OS cells. Preservation of SUMO-1 modifications requires rapid inhibition of SUMO-1-specific isopeptidases and this is accomplished by direct lysis of cells into buffer containing SDS and iodoacetamide. In the absence of any stimulus, p53 and more slowly migrating products, which we assume to be ubiquitin adducts, are detected. Also present is a species of 65 kDa, which is consistent with a single SUMO-1 addition to p53. Exposure of U2OS cells to UV irradiation results in a time-dependent increase in p53 levels and an accumulation of the 65 kDa species (Figure 1A). The lack of immunoreactive species in Saos-2 cells, which lack p53, indicates that the more slowly migrating species are indeed related to p53 (Figure 1B, right panel). To establish that the 65 kDa species is indeed the SUMO-1 modified form of p53, U2OS cells were transfected with a His$_6$ version of SUMO-1, either alone or with a p53 expression construct or empty vector as a control. Cells were lysed in guanidinium–HCl and His$_6$-containing proteins eluted from Ni-agarose with imidazole. Western blotting with an antibody to p53 indicated that His$_6$-SUMO-1 linked to p53 could be detected in cells transfected with the His$_6$-SUMO-1 expression plasmid, but not in cells transfected with the empty vector. Cotransfection with a p53 expression construct strongly increased the quantity of His$_6$-SUMO-1 modified p53 (Figure 1B). Treatment of His$_6$-SUMO-1 transfected U2OS cells with UV irradiation results in a time-dependent increase in the amount of His$_6$-SUMO-1 conjugated to p53 (Figure 1B, right panel). Thus, p53 is covalently coupled to SUMO-1

in vivo and this modified form of p53 accumulated after UV irradiation.

SUMO-1 modification of p53 takes place on K386

To investigate the site of SUMO-1 modification in p53 an *in vitro* system for SUMO-1 modification was employed (Desterro *et al.*, 1998). ^{35}S-labelled *in vitro*-translated p53 was incubated with a source of SUMO-1 activating enzyme (SAE1/2) (Desterro *et al.*, 1999) and SUMO-1 conjugating enzyme (Ubc9) (Desterro *et al.*, 1997) in the presence of SUMO-1 and ATP. Under these conditions ^{35}S-labelled p53 was converted to a more slowly migrating form, which is consistent with SUMO-1 modification. To confirm that this species was indeed SUMO-1 modified p53, GST–SUMO-1 was substituted for SUMO-1 in the reaction, resulting in the detection of a modified species with altered electrophoretic mobility (Figure 2A). SUMO-1 modification was abolished if either SUMO-1, SAE, Ubc9 or ATP was omitted from the reaction (Figure 2A). A series of p53 molecules in which regions of the N- and C-termini had been deleted (Hansen *et al.*, 1998) were used as substrates for SUMO-1 modification *in vitro* (Figure 2D). p53 molecules lacking parts of the N-terminus were efficiently SUMO-1 modified whereas p53 molecules lacking parts of the C-terminus were not modified by SUMO-1 (Figure 2B). Deletion 4M, which lacks the C-terminal 30 amino acids, was not modified thus indicating that this region is required for modification and may in fact be the site of modification. As ubiquitin-like proteins modify substrates on the ε-amino group of lysine, a series of mutants was created in which lysines within the C-terminal 30 amino acids of p53 were changed to arginine. Modification of K370, K372, K373, K381 or K382 was without consequence for SUMO-1 modification, whereas p53 containing the K386R mutation was not modified by SUMO-1 *in vitro* (Figure 2C). To establish that the *in vitro* results are an accurate representation of the situation *in vivo*, the same collection of p53 mutants was co-expressed with His_6-SUMO-1 in cells lacking endogenous p53. Proteins conjugated to His_6-SUMO-1 were isolated by chromatography on Ni-agarose and p53 detected by Western blotting. Again mutations in K370, K372, K373, K381 and K382 did not affect SUMO-1 modification, while the K386R mutation abolished SUMO-1 modification *in vivo* (Figure 3A). Direct Western blotting of the cell lysates before Ni-agarose chromatography indicated that each of the p53 proteins was expressed at comparable levels. Although representing only a small proportion of the total, due to overexpression of p53, the SUMO-1 modified forms of p53 proteins containing K386 are detected. This SUMO-1 modified form of p53 is not detected in those proteins containing the K386R mutation (Figure 3B). Thus, K386R is required for SUMO-1 modification and is likely to be the residue to which SUMO-1 is conjugated.

SUMO-1 and ubiquitin modification do not compete for the same lysine acceptor site in p53

It has recently been established (Kubbutat *et al.*, 1998; Honda and Yasuda, 1999) that the C-terminal 30 amino acids are required for Mdm2-mediated degradation of p53. In light of recent observations with IκBα the effect of SUMO-1 modification on p53 ubiquitylation was investigated. As expected, *in vitro* ubiquitylation of ^{35}S-labelled p53 was dependent on the presence of Ubc5 and Mdm2 (Figure 4A) and required the C-terminus of p53 (Figure 4B, 4M deletion). To determine if ubiquitylation takes place at the same site as SUMO-1 modification the same lysine to arginine point mutants (Figure 2) were used for *in vitro* ubiquitylation. Unlike SUMO-1 modification, K386R was competent for ubiquitylation and none of the other point mutations had a substantial effect on ubiquitylation. Thus, the SUMO-1 and ubiquitin modification systems do not compete for modification of the same lysine residue in p53.

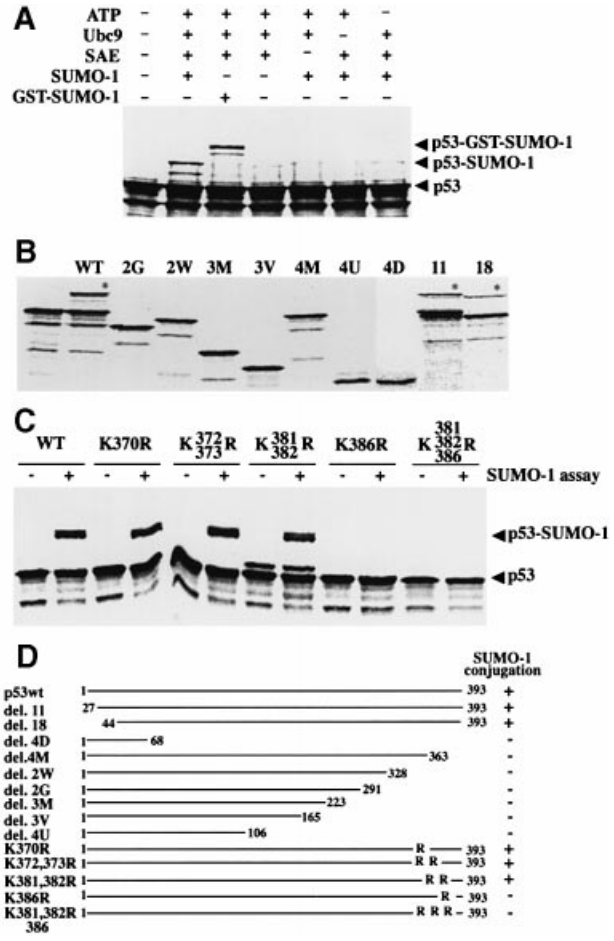

Fig. 2. The C-terminus of p53 is modified by SUMO-1 *in vitro*.
(**A**) Requirements for *in vitro*-conjugation of SUMO-1 to p53. *In vitro* expressed and ^{35}S-labelled p53 was incubated with ATP, recombinant SUMO-1 or GST–SUMO-1, Ubch9, and HeLa fraction II.4 containing SUMO-1 E1 activity (SAE) as indicated. Reaction products were fractionated by SDS–PAGE, and the dried gel was analysed by phosphorimaging. The positions of p53, the SUMO-1 conjugated form of p53 and the GST–SUMO-1 conjugated form of p53 are indicated. (**B**) *In vitro* SUMO-1 conjugation of C- and N-terminal p53 deletions. *In vitro* expressed and ^{35}S-labelled wild-type p53 (WT) and C- and N-terminal deletions were incubated with recombinant Ubch9, SUMO-1 and HeLa fraction II.4 containing SUMO-1 E1 activity. Reaction products were fractionated by SDS–PAGE, and the dried gel was analysed by phosphorimaging. The SUMO-1 conjugated form of p53 is indicated by an asterisk. (**C**) *In vitro* SUMO-1 conjugation of C-terminal lysine point mutants of p53. *In vitro*-expressed and ^{35}S-labelled WT p53 and mutants were assayed for SUMO-1 conjugation *in vitro* as in (B). The positions of p53 and the SUMO-1 conjugated form of p53 are indicated. (**D**) Schematic representation of p53 deletions, C-terminal lysine to arginine point mutants and their ability to act as substrates for SUMO-1 conjugation activity.

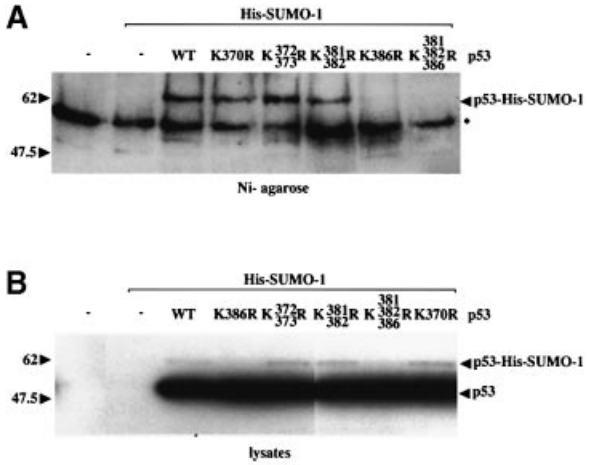

Fig. 3. K386 is required for SUMO-1 modification of p53 *in vivo*. (**A**) Saos-2 cells were transfected with the indicated plasmids and after 36 h of expression lysed in buffer containing guanidinium–HCl. His_6 linked proteins, eluted from Ni^{2+}-NTA-agarose, were fractionated by SDS–PAGE and analysed by Western blotting with anti-p53 antibody (DO-1). Molecular weight markers and the position of His-tagged SUMO-1 conjugated to p53 are indicated. An asterisk indicates a non-specific cross-reacting species, which is independent of transfection. (**B**) Saos-2 cells transfected as indicated in (A) above were directly analysed by Western blotting with anti-p53 antibody (DO-1).

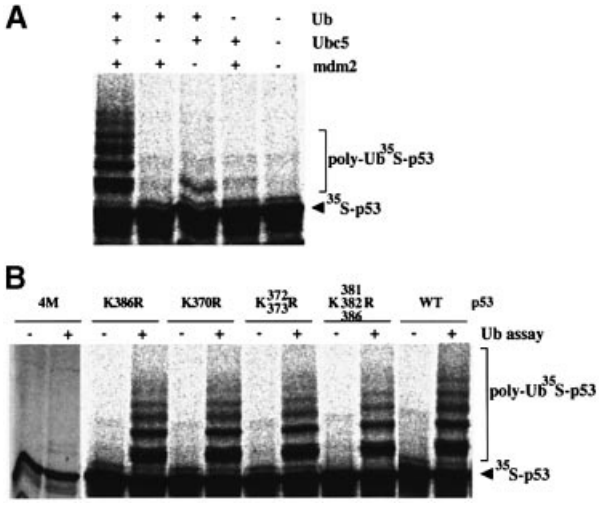

Fig. 4. SUMO-1 and ubiquitin modification do not compete for the same lysine residue in p53. (**A**) ^{35}S-labelled p53 generated by *in vitro* transcription and translation was incubated with recombinant E1 in the presence (+) or absence (–) of different components, as indicated in the figure, in 50 mM Tris pH 7.5, 5 mM $MgCl_2$ and an ATP regenerating system (see Materials and methods) at 37°C for 2 h. Reaction products were analysed as described in the legend to Figure 2B. (**B**) *In vitro* ubiquitylation of ^{35}S-labelled p53, the indicated p53 point mutants and the C-terminal deletion 4M was carried out as described in (A).

SUMO-1 modification induces p53-dependent transcription

To assess the functional consequences of SUMO-1 conjugation to p53 we determined the effect of cotransfected SUMO-1 on the transcriptional activity of wild-type p53 and K386R p53 where the SUMO-1 acceptor site has been mutated. Soas-2 cells, which lack endogenous p53, were cotransfected with the p53-responsive reporter plasmid pG13-luciferase and expression plasmids for p53

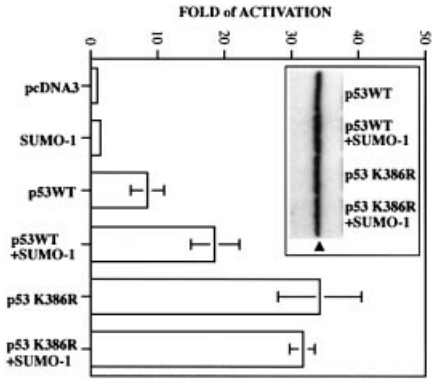

Fig. 5. SUMO-1 conjugation on K386 stimulates the transcriptional activity of p53. Saos-2 cells were transfected by electroporation with pG13-luc reporter plasmid and expression plasmids for SUMO-1, wild-type p53 and K386R p53 as indicated. Twenty-four hours after electroporation reporter activity was determined. Each point is the mean from four independent transfections with error bars representing one standard deviation. In the insert is shown a representative experiment in which p53 levels were determined by Western blotting using antibody DO-1.

and SUMO-1. Wild-type p53 alone activates transcriptional activity 8-fold while the additional presence of SUMO-1 activates transcription by ~18-fold (Figure 5). In contrast K386R p53 has an inherently higher transcriptional activity and this is not further augmented by coexpression of SUMO-1 (Figure 5). Similar data were obtained over a wide range of input p53 expression vector amounts and with different p53-dependent reporters, such as the mdm2 promoter (data not shown). Western blotting indicated that neither wild-type p53, nor K386R p53 levels were dramatically altered by coexpression of SUMO-1 (Figure 5, insert).

Discussion

In normal cells p53 is maintained at a low level by mdm2-mediated ubiquitin-dependent proteolysis. Following genotoxic insult p53 is stabilized and as a consequence the protein accumulates and activates p53-dependent transcription. As no single unifying hypothesis can yet describe this phenomenon, it is likely that multiple pathways lead to activation of the p53 response (Lane, 1998). Here we demonstrate that p53 is modified by SUMO-1 and that the amount of SUMO-1 conjugated p53 increases after exposure of cells to UV irradiation (Figure 1). SUMO-1 is conjugated to p53 on K386 (Figures 2 and 3), which is located in the C-terminus of the protein in a region that is known to be required for mdm2-mediated ubiquitin-dependent degradation of p53 (Kubbutat *et al.*, 1998; Honda and Yasuda, 1999). We proposed previously (Desterro *et al.*, 1998) that efficient SUMO-1 modification takes place at a sequence ψKxE, which is present at sites of modification in RanGAP1, IκBα and PML. The sequence FKTE, which is modified in p53, also conforms to this consensus, suggesting that ψKxE represents a recognition site for the SUMO-1 conjugation machinery. A role for SUMO-1 modification in p53 activation is consistent with the observation that overexpression of SUMO-1 leads to increased p53-dependent transcriptional activity. This increase is dependent on SUMO-1 modification of p53, as SUMO-1 overexpression does not influence

the transcriptional activity of K386R p53 (Figure 5). It is possible to envisage a number of models by which SUMO-1 modification may increase the transcriptional response of p53. These include an increase in protein stability, a higher intrinsic transcriptional activity and alterations in subcellular localization. Although the site of SUMO-1 modification may represent a potential site of ubiquitylation, mutation K386R does not have a dramatic effect on ubiquitylation *in vitro* (Figure 4). While the C-terminal region is required for ubiquitylation it does not appear that a unique lysine is a target for mdm2 as no single lysine to arginine mutation of the six lysines in this region abolishes ubiquitylation (Figure 4). This is different from the situation in IκBα where one of two lysine residues is the target for signal-induced ubiquitination. As this also represents the site of SUMO-1 conjugation, ubiquitylation is inhibited simply because the acceptor lysine is already modified by SUMO-1 (Desterro *et al.*, 1998). Although the major binding site for mdm2 is located in the N-terminal region of p53 (Momand *et al.*, 1992; Chen *et al.*, 1993; Oliner *et al.*, 1993; Picksley *et al.*, 1994; Kussie *et al.*, 1996; Bottger *et al.*, 1997; Midgley and Lane, 1997) it has also been reported that the oligomerization of p53 is required for efficient interaction with mdm2 (Marston *et al.*, 1995). While the extreme C-terminus of p53 is not required for efficient interaction with mdm2, its deletion dramatically reduces mdm2-catalyzed ubiquitylation *in vitro* and increases the stability of p53 *in vivo* (Kubbutat *et al.*, 1998; Honda and Yasuda, 1999). It is possible that the C-terminal region of p53 is required as the site of ubiquitylation by mdm2, but ubiquitylation can be targeted to any of the six lysines in this region. Thus, while SUMO-1 modification of this region may not directly disrupt the interaction between p53 and mdm2, it could inhibit the ability of mdm2 to access the lysine residues in the C-terminus, which are the substrates for ubiquitylation. In addition to SUMO-1 modification, it has recently been reported that phosphorylation of p53 with DNA-dependent protein kinase reduces the substrate activity of p53 in an mdm2-dependent *in vitro* ubiquitylation assay (Honda and Yasuda, 1999). The tumour suppressor $p19^{ARF}$ blocks ubiquitylation of p53, but in this case the target appears to be mdm2 rather than p53 (Honda and Yasuda, 1999). Likewise, the retinoblastoma gene product (RB) has also been shown to block mdm2-induced degradation of p53 and in this instance a trimeric, but inactive complex containing p53, RB and mdm2 was detected (Hsieh *et al.*, 1999). As an alternative to effects on protein stability, SUMO-1 modification could increase the inherent transcriptional activity of p53. This could result from a change in protein structure that increases DNA-binding activity, allows more efficient access to p53-dependent promoters embedded in repressive chromatin, or has a greater capacity to recruit co-activators such as CBP/p300. In this respect K386R p53 elicits a greater transcriptional response than wild-type p53, which may reflect an increased activity in any one of the above properties.

It is also possible that SUMO-1 modification alters the subcellular localization of p53, as has been noted for other SUMO-1 modified proteins. SUMO-1-modified Ran GAP is recruited to the nuclear pore complex (Matunis *et al.*, 1996; Mahajan *et al.*, 1997) while SUMO-1 modified PML and SP100 are present in PML oncogenic domains (PODs; Sternsdorf *et al.*, 1997; Muller *et al.*, 1998; Duprez *et al.*, 1999). IκBα is predominantly cytoplasmic but shuttles rapidly between the nucleus and the cytoplasm, and while the cellular localization of SUMO-1 modified IκBα has not been determined, it is worth noting that IκBα, trapped in the nucleus by leptomycin B, is resistant to signal-induced degradation (Rodriguez *et al.*, 1999). Mdm2 also shuttles between the nucleus and the cytoplasm (Roth *et al.*, 1998) and blockage of nuclear export results in accumulation of p53–mdm2 complexes in subnuclear domains adjacent to the PODs (Lain *et al.*, 1999), which are rich in SUMO-1 modified proteins. Like IκBα, nuclear p53 is resistant to degradation (Freedman and Levine, 1998) and it is possible that SUMO-1 modification takes place when these proteins are imported into the nucleus. The predominantly nuclear localization of the SAE and Ubc9 enzymes involved in SUMO-1 modification (M.S. Rodriguez, J.M.P.Desterro and R.T.Hay, unpublished observations) lends support to this hypothesis. Thus, SUMO-1 modification could increase the nuclear content of p53 or alternatively concentrate the protein in distinct subnuclear domains that are active sites of transcription. Diverse stress stimuli activate the p53 response and are likely to act via separate signalling pathways with distinct activation mechanisms. SUMO-1 modification represents an additional route to p53 activation that may link the p53 response to other aspects of cellular metabolism. Manipulation of the SUMO-1 modification pathway may therefore represent a novel target for the development of therapeutically useful modulators of the p53 response.

Materials and methods

Ubiquitin was purchased from Sigma. Monoclonal antibody (mAb) DO-1 (Vojtesek *et al.*, 1995) recognizes only human p53 and the polyclonal rabbit serum CM-1 was raised against human p53 expressed in *Escherichia coli* (Midgley *et al.*, 1992).

Plasmid construction

PCR was used to generate a wild-type version of the p53 coding sequence containing a *Bam*HI site at the sequence corresponding to G361/S362. Full-length p53 was cloned into *Hin*dIII–*Eco*RI sites of pcDNA3 vector. All p53 mutants were generated by site-directed mutagenesis using PCR strategy. The wild-type p53 *Bam*HI–*Eco*RI cassette was replaced to generate each K to R mutant. DNA sequence was determined by University of St Andrews DNA sequencing facility (ABI377). DNA encoding six histidines was inserted into pcDNA3 using *Kpn*I and *Bam*HI cloning sites and the following oligonucleotides: 5′-CATGGCTCATCATCATCATCATGGTG-3′ and 5′-GATCCA-CCATGATGATGATGATGATGAGCCATGGTAC-3′. SUMO-1 cDNA (Desterro *et al.*, 1997) was inserted into the *Bam*HI cloning site of the constructed pcDNA3-His_6-N vector.

Cell culture and transfections

Wild-type p53 U2OS and p53 null Saos-2 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected by electroporation (950 mF, 200 V, Equibio Easyject plus) as described (Arenzana-Seisdedos *et al.*, 1997). A total of 10 μg of plasmid DNA encoding the His_6–SUMO-1 and p53 were co-transfected into 5×10^6 cells. After transfection, cells were seeded in 75 cm^3 flasks and incubation continued for a further 36 h. To measure p53 transcriptional activity, 10^6 Saos-2 cells were electroporated with 1 μg of pG13-luc reporter plasmid together with 1 μg of SUMO-1 expression plasmid and 1 ng of p53 (wild-type and K386R) expression plasmid. pcDNA3 empty vector was used to bring the total amount of DNA to 10 μg. After electroporation cells were grown in 6-well plates for 24 h and processed for luciferase reporter activity as described (Rodriguez *et al.*, 1996). Results are the

mean of four independent transfections, with error bars representing one standard deviation.

Western blotting

Cell extracts were lysed in SDS sample buffer (5% SDS, 0.15 M Tris–HCl pH 6.7, 30% glycerol) diluted 1:3 in RIPA buffer (25 mM Tris pH 8.2, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.1% azide), containing 10 mM iodoacetamide and complete® protease inhibitor cocktail (Boehringer Mannheim). Lysates were sonicated briefly and cleared by centrifugation. Protein (20 μg) from total cell lysate was fractionated by electrophoresis on 10% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Sigma). Western blotting was performed using ECL detection system. Horseradish peroxidase conjugated anti-mouse IgG and anti-rabbit IgG (Amersham) were used as secondary antibodies.

Expression and purification of recombinant proteins

SUMO-1, Ubch5 and Ubch9 were expressed and purified as reported (Desterro *et al.*, 1997). Human E1 ubiquitin-activating enzyme was purified from recombinant baculovirus-infected insect cells as described (Rolfe *et al.*, 1995). Human Mdm2 (residues 6–491) was expressed in bacteria and purified as described for p53 (Midgley *et al.*, 1992).

Purification of His_6-tagged SUMO-1 p53 conjugates

Thirty-six hours after transfection cells were lysed in 4 ml of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris–HCl pH 8.0 plus 5 mM imidazole and 10 mM β-mercaptoethanol per 75 cm^3 flask. After sonication, to reduce viscosity, the lysates were mixed with 50 μl of Ni^{2+}-NTA-agarose beads prewashed with lysis buffer and incubated for 2 h at room temperature. The beads were successively washed with the following: 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris–HCl pH 8.0 plus 10 mM β-mercaptoethanol; 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris–HCl pH 8.0, 10 mM β-mercaptoethanol; 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris–HCl pH 6.3, 10 mM β-mercaptoethanol (buffer A) plus 0.2% Triton X-100; buffer A and then buffer A plus 0.1% Triton X-100. After the last wash with buffer A the beads were eluted with 200 mM imidazole in 5% SDS, 0.15 M Tris–HCl pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol. The eluates were subjected to SDS–PAGE (10%) and the proteins transferred to a PVDF membrane (Sigma). Western blotting was performed with a mAb against p53, DO-1.

In vitro expression of proteins

In vitro transcription/translation of proteins was performed using 1 μg of plasmid DNA and a wheat germ coupled transcription/translation system according to the instructions provided by the manufacturer (Promega). [^{35}S]methionine was used in the reactions to generate radiolabelled proteins.

In vitro SUMO-1 conjugation assay

[^{35}S]methionine-labelled *in vitro* transcribed/translated p53 proteins (1 μl) were incubated with 2 μl of HeLa cell fraction containing SUMO-1 E1 activity (Fr II.4), (Kroll *et al.*, 1997) in a 10 μl reaction including an ATP regenerating system (50 mM Tris pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase and 0.6 U/ml of inorganic pyrophosphatase), 10 μg SUMO-1, 600 ng Ubch9. Reactions were incubated at 37°C for 2 h. After terminating the reactions with SDS sample buffer containing mercaptoethanol, reaction products were fractionated by SDS–PAGE (10%) and the dried gels analysed by phosphorimaging (Fujix BAS 1500, MacBAS software).

In vitro ubiquitylation assay

[^{35}S]methionine-labelled *in vitro* transcribed/translated p53 proteins (1 μl) were incubated in a 10 μl reaction including an ATP regenerating system (50 mM Tris pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase and 0.6 U/ml of inorganic pyrophosphatase), 10 μg ubiquitin, 10 ng human E1, 60 ng Ubch5 and 60 ng Mdm2. After incubation at 37°C for 2 h the reaction products were treated as described for the SUMO-1 conjugation assay.

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