Covalent Modification of p73α by SUMO-1

TWO-HYBRID SCREENING WITH p73 IDENTIFIES NOVEL SUMO-1-INTERACTING PROTEINS AND A SUMO-1 INTERACTION MOTIF

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Two-hybrid screening in yeast with p73α isolated SUMO-1 (small ubiquitin-like modifier 1), the enzyme responsible for its conjugation, Ubc-9, and a number of novel SUMO-1-interacting proteins, including thymine DNA glycosylase, PM-Scl75, PIASx, PKY, and CHD3/ZFH. A subset of these proteins contain a common motif, hhX-SX5/Taaa, where h is a hydrophobic amino acid and a is an acidic amino acid, that is shown to interact with SUMO-1 in the two-hybrid system. We show here that p73α, but not p73β, can be covalently modified by SUMO-1. The major SUMO-1-modified residue in p73α is the C-terminal lysine (Lys⁶⁰⁷). The sequence surrounding this lysine conforms to a consensus SUMO-1 modification site b(X)XhXXE, where b is a basic amino acid. SUMO-1-modified p73 is more rapidly degraded by the proteasome than unmodified p73, although SUMO-1 modification is not required for p73 degradation. SUMO-1 modification does not affect the transcriptional activity of p73α on an RGC-luciferase reporter gene in SK-N-AS cells. Instead, SUMO-1 modification may alter the subcellular localization of p73, because SUMO-1-modified p73 is preferentially found in detergent-insoluble fractions. Alternatively, it may modulate the interaction of p73 with other proteins that are substrates for SUMO-1 modification or which interact with SUMO-1, such as those identified here.

Covalent modification of proteins is widely used as a way of modifying their stability, activity, or localization. Examples of this are phosphorylation, acetylation, lipid modification, or glycosylation. Modification by covalent linkage to a second “tagging” protein was first observed with ubiquitin, a 76-amino acid polypeptide that is covalently linked to lysine residues in an acceptor protein by an enzymatic system involving two to three ubiquitin-activating and -conjugating enzymes (E1, E2, and E3). Subsequent poly-ubiquitination usually signals the modified protein for degradation by the proteasome (1). Alternate outcomes for ubiquitinated proteins are activation or transport via an intracellular membrane vesicular system (2).

It has now become apparent that several other “ubiquitin-like” tagging molecules exist that are conjugated using enzymatic systems similar but nonidentical to those used by ubiquitin (3, 4). Two groups initially determined the nature of a modification of the Ran GTPase-activating protein (RanGAP1) involved in the interaction of this protein with RanBP2/Nup358 at the nuclear pore complex (5, 6). They called the modifying molecule GMP1 (GAP-modifying protein 1) or SUMO-1 (small ubiquitin-like modifier 1). SUMO-1 has since been identified several times as an interacting partner in the yeast two-hybrid system and given different names: with the promyelocytic leukemia gene product (PML) (PIC-1) (7), with the death domain of the FAS antigen or TNF-receptor (sentrin, DAP-1) (8, 9), and with the RAD51 and RAD52 proteins involved in DNA recombination and repair (UBL-1) (10).

A budding yeast homologue of SUMO-1 (Smt3p) was identified by Meluh and Koshland (11) as a suppressor of mutations in Mig2 (mitotic instability factor 2), a protein thought to be the yeast equivalent of the CENP-C mammalian centromere protein. One putative role for SUMO-1/Smt3p is thus in assembly or maintenance of the centromere/kinetochore structure involved in chromosome segregation. Similarly, the fission yeast (Schizosaccharomyces pombe) equivalent of Smt3p, Pmt3p, has recently been shown to be involved in chromosomal segregation and the control of telomere length (12).

Enzymes involved in Smt3p/SUMO-1 conjugation have been identified in yeast and mammalian cells (13–15). The SUMO-1-activating enzyme (E1) consists of a heterodimer of the Aos1p/SAE1 and Uba2p/SAE2 proteins that together reconstitute the equivalent of the ubiquitin-activating enzyme Uba1 (13). The SUMO-1-conjugating enzyme (E2) in yeast, mammalian cells, and Xenopus is Ubc9 (14, 15). So far no E3 enzymes have been identified. Ubc9, originally thought to be a ubiquitin-conjugating enzyme, was shown to be essential gene in yeast (16) because temperature-sensitive mutants of Ubc9 arrested in mitosis, as did mutants of a SUMO-1-cleaving protease Ulp1 (17).

In yeast and mammalian cells, there is very little free Smt3p/SUMO-1. Most (>90%) of the SUMO-1 detected on Western analysis of extracts from mammalian cells is conjugated to the RanGAP1 nuclear pore protein (18). Other proteins subject to modification by SUMO-1 are the PML and Sp100 proteins, which form part of the nuclear structures known as PODs (PML oncogenic domains) or nd10s (19). For PML, it has been shown that SUMO-1 modification is essential for its localization in PODs, with free PML being found in the nucleoplasm. Another substrate for SUMO-1 is Ikβα (20), an inhibitor of NFκβ, which is modified by SUMO-1 on the lysine residue also modified by ubiquitin. SUMO-1 modification prevents ubiquitination and thus results in stabilization of Ikβα and consequently in inhibition of NFκβ (20).

In the present communication, we describe SUMO-1 modification of the p53-related p73α protein. p53 is the most widely
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studied tumor suppressor and is mutated in over 50% of human tumors (21). It plays a key role in both the regulation of cell cycle checkpoints and the initiation of apoptotic cell death in response to DNA damage. The activity of p53 has been shown to be finely tuned by a variety of post-translational modifications (phosphorylation, acetylation, and glycosylation) and to be highly sensitive to conformational changes (22). The p53 molecule contains a number of well defined domains including an N-terminal transcriptional activation domain and a central core, corresponding to the DNA-binding domain, which is highly conserved in evolution and which contains the majority of the mutation hot spots in cancer cells. The rest of the molecule contains a linker region including the major nuclearization signal, an oligomerization domain, and a regulatory C-terminal region containing multiple phosphorylation and acetylation sites (21, 22). Recently, this region has been shown to contain a lysine residue (386) that can be covalently modified by SUMO-1 (23, 24).

We previously reported the existence of a p53-related gene, p73, mapping to a chromosomal locus (1p36.3) often deleted in neuroectodermal human cancers such as neuroblastomas (24). Subsequent work from several laboratories has described the existence of another p53 family member, more closely related to p73 than to p53, variously described as p63, KET, and p51 (25). p73 shows structural similarities with p53, including the presence of transcription-activating, DNA-binding, and oligomerization domains. It exists as multiple isoforms, resulting from differential splicing of C-terminal exons, of which the two major forms are the α and β isoforms containing 636 and 499 amino acids (25, 26). p73 differs from p53 in that its levels of expression are not elevated in response to environmental stresses such as UV irradiation and actinomycin D treatment (25). However, in interaction with the protein kinase c-Abl it mediates an apoptotic response to ionizing radiation and to genotoxic agents such as cisplatin (27).

In contrast to p53, no functionally significant p73 mutations have so far been reported in cancer cells, but a monoallelic pattern of expression is sometimes observed (26). Analysis of p73 knockout mice does not show an increased susceptibility to spontaneous tumorigenesis (28). However, these studies reveal that p73 plays key roles in a number of developmental processes that are nonoverlapping with the roles played by other p53 family members (28). The activities of p73 may be exerted at multiple levels. Firstly, p73 is a transcriptional activator eliciting a response different from that obtained with p53 (29). Secondly, the major form of p73 is often an N-terminally truncated form that would be incapable of transcriptional activation (28), suggesting the possibility of other nontranscriptional roles for p73.

During yeast two-hybrid screens using p73 as a bait, we isolated the cDNA for SUMO-1. Here we show that p73 can be covalently modified by SUMO-1, with the major modification occurring on the terminal lysine residue. A number of other SUMO-1-interacting proteins were isolated in the p73 two-hybrid screening, and we have been able to deduce and confirm a novel SUMO-1 interaction motif. The nature of the proteins identified here suggests that one role for SUMO-1 may be in transcriptional regulation, perhaps co-ordinating this with other cellular processes such as cell cycle checkpoints, chromosome segregation, DNA recombination and repair, and the induction of apoptosis.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The SK-N-AS neuroblastoma cell line (30) and the 293 embryonic kidney cell line (American Type Culture Collection CRL 1573) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 1 mM sodium pyruvate and 10% fetal calf serum. The U937 monocytic cell line (American Type Culture Collection CRL 1593) was grown in RPMI (Life Technologies, Inc.) containing 10% fetal calf serum.

RNA Preparation and cDNA Library Construction—Total cellular RNA was extracted from SK-N-AS and U937 cells by the guanidinium thiocyanate-phenol method (31). Poly(A)+ RNA was isolated using oligo(dT) magnetic beads (Dynal). 1 µg of each poly(A)+ RNA was transcribed into cDNA using reverse transcriptase (Superscript, Life Technologies, Inc.) and the primer GATCCGGGCGATCTTTTGTACCGT(ACGGT)(ACGGT)(ACGGT)(ACGGT). cDNAs were fractionated on Sephacryl S400 (Amersham Pharmacia Biotech), and fractions containing cDNA of approximately 500–1500 nucleotides were selected for cloning. The plasmid pJGC cloning vector, derived from the pEG202 bait plasmid (32) by insertion of a polylinker containing Apol and BamHI cloning sites between the EcoRI and HindIII sites, was used for cloning following isolation of the cDNA library preparations. p73 was amplified using primers containing the LexA DNA-binding domain. The pEG202.p73 bait was amplified using lithium acetate/ polyethylene glycol transformation with sheared single-stranded DNA carrier (34) into the EGY48 strain of Saccharomyces cerevisiae (containing the LEU2 gene under the control of six LexA operators) along with a modified pSH18-34 containing the LexA gene under the control of eight LexA operators. cDNA libraries were then similarly introduced, and yeast colonies were selected on Yeast Nitrogen Base (Difco) medium containing 2% glucose and leucine (but lacking tryptophan, histidine, and uracil). Approximately 106 transformed yeast were obtained with the U937 and 2 × 107 transformed yeast with the SK-N-AS. After 3–4 days, colonies were replicated to nitrocellulose filters (Protran BA85; Schleicher & Schull), replated on plates containing 2% galactose, 1% raffinose, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-gal) (Life Technologies, Inc.) lacking leucine, and grown for 4–5 days. 20 yeast colonies from each cDNA library transformation showing a blue coloration were selected for further study.

Plasmid Identification—Plasmid DNA was extracted using a glass bead disruption method (35), and cDNA inserts in the pJGC plasmid were amplified by polymerase chain reaction using oligonucleotides flanking the cDNA insert and sequenced. The pJGC plasmid was isolated by selection in the KC8 bacterium in minimal A medium containing vitamin B1 and supplemented with uracil, histidine, and leucine but lacking tryptophan. It was then tested for interaction with the p53 and p73 bait plasmids by measurements of the ß-galactosidase levels in transformed EGY48 24 h after galactose induction of the GAL1 promoter in pJGC, as described by Kippert (36).

Site-directed Mutagenesis—Amino acid substitutions were performed by limited polymerase chain reaction amplification of plasmid DNA using Pfu DNA polymerase and oligonucleotides containing the mutated codons, followed by digestion of remaining input plasmid DNA using the methylation sensitive enzyme DpnI (QuickChange; Stratagene).

Transient Transfection of Animal Cells—The p73α, p73β, p53, and SUMO-1 cDNAs were introduced into the pdeg3A3 vector (Invitrogen) or an epitope-tagged vector derived from pdeg3A3 by insertion of an optimized ATG codon (CCACCATGGCG) and a c-Myc 9E10 epitope (EQKLISEEDL) between the HindIII and EcoRI sites. Plasmid DNA preparations were performed using the QIAfilter Plasmid Midi Kit (Qiagen). Approximately 106 cells were transfected in six-well dishes using 1–2 µg of plasmid DNA and LipofectAMINE Plus reagents (Life Technologies, Inc.) as described by the manufacturer. Cells were scraped from the dish 20–30 h after transfection, resuspended in de-naturing SDS gel buffer (Bio-Rad) with 0.7 M β-mercaptoethanol and analyzed on SDS-polyacrylamide gels.

Immunoblotting and Antibodies—Proteins were transferred from polyacrylamide gels to nitrocellulose membranes (Hybond-C-extra; Amersham Pharmacia Biotech). These were analyzed with the following primary antibodies: anti-c-Myc (9E10) (Santa Cruz or Invitrogen), anti-GMP1 (anti-SUMO-1) (Zymed Laboratories Inc.), anti-p73α (a rabbit polyclonal antibody; (25)), anti-PCNA (Santa Cruz), and secondary antibodies with goat anti-rabbit IgG coupled to horseradish peroxidase (Transduction Laboratories) and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). The anti-p73 antibody was generated against a C-terminal p73α (427–636) glutathione S-transferase fusion protein (25). p73 forms were quantified by scanning of different film exposures and analysis using BioImage (Kodak) software.

Dual Luciferase Assays—SK-N-AS cells were transfected in six-well
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RESULTS

Two-hybrid Screening with p73α Isolates SUMO-1 and SUMO-1-interacting Proteins—While performing two-hybrid screens using a p73α protein sequence fused to the LexA DNA binding domain (32), we isolated cDNAs encoding SUMO-1, Ubc9 (the SUMO-1-conjugating enzyme), and a SUMO-1-interacting protein. These were normalized to that of the initial interaction of the PM-Scl75 protein with SUMO-1. These were normalized to that for the initial interaction of the PM-Scl75 protein. A similar motif from the protein furin (43) was also tested (sequence B).

Indeed, when tested directly with a SUMO-1 bait, all of these proteins gave a positive result (Fig. 1B).

A SUMO-1 Interaction Motif—When the cDNA sequences of the SUMO-1-interacting proteins were examined for the presence of common sequences, an 11-amino acid motif was detected in a subset of the proteins. This contained a central serine doublet separated by one amino acid (SXXS), which one serine was replaced by threonine in the human PKY cDNA (Fig. 2A). This SXXS triplet is flanked on the N-terminal side by acidic C-terminal residues and on the C-terminal side by acidic amino acids (D/E) (Fig. 2A). This motif is evolutionarily conserved in the PKY and PIAS gene families (Fig. 2A). In view of the subsequent mutagenesis experiments (see below), it is unclear whether the motif in SAE2 that served to derive this consensus would in fact be sufficient on its own to interact with SUMO-1.

The motif from the PM-Scl75 protein was used to construct a LexA-SXXS motif fusion protein, which showed a very strong interaction with SUMO-1 in the two-hybrid system (equivalent to that of the dimerization of p53/p53 and stronger than the initial PM-Scl75/SUMO-1 interaction). Critical residues were identified by alanine replacement. Such an analysis shows that both serine residues are necessary (Fig. 2B, sequences c and d). As is the one amino acid spacing between these residues; either no or two amino acid spacing destroys SUMO-1 interaction (Fig. 2B, sequences e and f). The acidic C-terminal residues are
also crucial. Mutation of E8 or E10 completely destroys SUMO-1 interaction (Fig. 2B, sequences g and i), and mutation of E9 drastically reduces interaction. (Fig. 2B, sequence h).

Although expression levels of the different mutants were not tested, it seems unlikely that differential expression of the mutant proteins (corresponding to single amino acid substitutions, additions or deletions) can explain the almost complete loss of the capacity to interact with SUMO-1. The SXS motif resembles one previously identified in the C terminus of the endopeptidase furin, which is implicated in its translocation into the trans Golgi network (43). However, the furin motif, which also has an acidic N terminus, does not interact with SUMO-1 (Fig. 2B, sequence b), indicating the potential importance of the hydrophobic residues (1–4) in the SXS motif.

**SUMO-1 Modification of p73**

*p73 Can Be Covalently Modified by SUMO-1*—We tested whether p73 might be covalently modified with SUMO-1 by co-transfection of SK-N-AS neuroblastoma cells with p73α and SUMO-1. In the presence of p73 and SUMO-1, a prominent novel protein species was formed, showing a molecular mass approximately 20 kDa more than p73α, both for the full-length and N-terminally truncated forms of p73α (Fig. 3, lanes b and d). This corresponds to the apparent molecular size difference for SUMO-1-modified forms of RanGAP1 and PML seen on SDS-polyacrylamide gel electrophoresis (5, 6, 18). A similar high molecular mass endogenous p73 species was observed in cell and tissue extracts, such as those from primary cultures of epithelial cells, isolated from human nasal polyps (a cell extract provided by Dr. F. Tournier, University of Paris VII) (44) (Fig. 3, lane f).

In addition to the major modified species (Fig. 3, lane h, indicated with **) several minor higher molecular mass species were seen in the p73 transfected cells (Fig. 3, lane h, indicated with *), which were of variable intensity from one experiment to another (Fig. 3, lanes b and h, and Fig. 4, lane a). The fact that the lysine in ubiquitin used for polyubiquitination is absent from SUMO-1 suggests the possibility of multiple sites for SUMO-1 modification.

The **Principal SUMO-1 Modification Site in p73 Is the C-terminal Lysine**—When p73α and p73β were transfected into SK-N-AS cells, SUMO-1-modified forms were detected for p73α but not for p73β (Fig. 3, lanes h and i). Similarly, after C-terminal deletion up to amino acid 450, p73 no longer showed SUMO-1 modification (Fig. 3, lane g). After deletion of the last 18 amino acids of p73α, no major SUMO-1-modified form was seen, but minor forms were still apparent (Fig. 3, lane j).

The major modification site in the last 18 amino acids of p73α was identified by site-directed mutagenesis as being the final lysine residue, number 627 (Fig. 4A). Similar experiments on p53 identified the C-terminal lysine residue (Lys<sup>386</sup>) as being the major SUMO-1 modification site (23, 24). In agreement with all but one of the previously identified SUMO-1 modification sites, the p53 lysine 386 and the p73α lysine 627 have a glutamic acid at +2 and a hydrophobic amino acid at position –1 (Fig. 4B). In addition, as suggested by Sternsdorf et al. (45), a basic residue (arginine, lysine, or histidine) is found at position –4 or –5 (Fig. 4B). A similar consensus sequence is found for the C-terminal lysine of p63α (Fig. 4B).

**SUMO-1 Modification Potentiates but Does Not Dictate p73α Instability**—Lee and La Thangue (46) reported that p73α and
Quantification of the modified and unmodified p73 forms in several experiments showed that the amount of SUMO-1-modified p73 is increased to a greater extent in the presence of MG132 (5–10-fold) than that of the unmodified p73 (1.5–3-fold) (Fig. 5A and B). Although the interconvertibility of the two p73 forms complicates the analysis, this result would suggest that SUMO-1 modification potentiates proteasomal degradation of p73.

**SUMO-1 Modification May Alter p73 Localization**—When performing detergent extractions using RIPA (1% Nonidet P-40, 0.5% sodium deoxycholate) buffer to prepare cell extracts, we often found that the SUMO-1-modified form of p73 was preferentially recovered in the detergent insoluble pellet fraction (Fig. 5B), whereas the nonmodified p73 was found in both soluble and pellet fractions. Treatment of cells with MG132 leads primarily to an increase in p73 accumulation in the pellet fraction (Fig. 5B). In contrast, the SUMO-modified form of RanGAP1 (Fig. 5C), which accumulates in nuclear pore complexes, and other nuclear proteins such as PCNA (Fig. 5D) are very efficiently extracted in the detergent-soluble fraction.

The preferential recovery of SUMO-1-modified p73 in the insoluble fraction may thus result from targeting of p73 modified by SUMO-1 to particular subcellular structures, although SUMO-1 modification is not required for the presence of p73 in this fraction because the mutant p73K627R distributes in a similar fashion to unmodified wild-type p73 (not shown). Alternatively, it may represent differential SUMO-1 modification of p73 in different cellular compartments or differential SUMO-1 cleavage during extraction. We have attempted to reduce isopeptidase cleavage during extraction by the addition of protease inhibitor mixtures. In addition, in certain experiments N-ethylmaleimide was included at concentrations from 10 nM to 1 μM in both washing and extraction buffers to inhibit the thiol protease activities reported for SUMO-1 hydrolases (47). This addition did not affect the preferential recovery of SUMO-1-modified p73 in the pellet fraction (Fig. 5E).

**SUMO-1 Modification Does Not Affect the Transcriptional Activity of p73**—To test the potential modulation of the transcriptional activities of p73α, we measured the activation of an RGC-luciferase reporter gene in SK-N-AS cells by different p73 forms in the presence or absence of SUMO-1. As can be seen in Fig. 6, the level of activation of the reporter gene by p73α is not affected by co-transfection with a large excess of a SUMO-1 expressing plasmid in these experiments. A similar result was found for activation by p53 (not shown). The transcriptional activities of p73α and of the mutant p73α K627R in these experiments are equivalent and are lower than that of p73β (Fig. 6).

**DISCUSSION**

p73α Is Modified by SUMO-1, but p73β Is Not—We show here that p73α is a novel substrate for SUMO-1 modification. It is of interest that of the two p73 isoforms (which differ only in their C termini), p73α is a good substrate for SUMO-1 modification, whereas p73β is not. Accordingly, deletion experiments show that the lysines involved in SUMO-1 modification are contained in the C-terminal region of p73α not present in p73β. It remains to be determined whether p73α is also a substrate for modification by SUMO-2/3 (48).

p73α shows one major SUMO-1-modified form and several minor modified forms. Because the lysine in ubiquitin used for polyubiquitination (lysine 48) is absent from SUMO-1, this suggests that there is one major site and several minor sites for SUMO-1 modification. Site-directed mutagenesis shows that the major modification site of p73α is the extreme C-terminal lysine. Similarly, the C-terminal lysine residue of p53 (lysine 386) has recently been identified as the major SUMO-1 modi-
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Fig. 6. Lack of effect of SUMO-1 on the transcripional activity of p73 in SK-NS neuroblastoma cells. SK-NS cells were co-transfected with the RGC-luciferase gene (200 ng), the CMV-Renilla luciferase gene (100 ng), a plasmid expressing a p73 isoform (α, β, αK627R) (5, 50, or 200 ng), with 1 μg of either the SUMO-1 expression plasmid or the equivalent vector without cDNA insert. After 20 h, cell lysates were prepared and activities of the firefly (RGC) and Renilla plasmid or the equivalent vector without cDNA insert. After 20 h, cell lysates were prepared and activities of the firefly (RGC) and Renilla luciferases measured. Shown are the means and ranges of values for three experiments for the RGC-luciferase, normalized to that of the Renilla luciferase, and then further normalized to the maximum 100% value for p73α in each experiment.

SUMO-1 Modification in Regulating the Stability or Localization of p73α—Ubiquitin modification is primarily, though not exclusively, involved in regulating protein stability, including that of p53 (1, 2). We investigated whether SUMO-1 modification plays a role in modulating the stability of p73, because Lee and La Thangue (46) reported that p73α is sensitive to degradation by the proteasome, whereas p73β is not. Our results, using an inhibitor of proteasomal degradation, MG132, show that both SUMO-1-modified and nonmodified p73α are degraded via the proteasome. p73α mutated in the major SUMO-1 modification site and wild-type p73α show similar up-regulation by MG132 treatment. SUMO-1 modification would thus not seem to be a major factor influencing p73α degradation by the proteasome. However, SUMO-1-modified p73α was stabilized to a greater extent than unmodified p73α by treatment with MG132, suggesting that SUMO-1 modification potentiates proteasomal degradation. Although some proteins have been shown to be stabilized by SUMO-1 modification because of a resulting inhibition of ubiquitination on the modified lysine residue (20, 50), other SUMO-1-conjugates have been shown to be degraded via the proteasome (51, 52). SUMO-1 modification may induce conformational changes potentiating ubiquitination or may influence protein degradation via modulation of E3 ubiquitin ligases, as found for the ubiquitin-like modifier Rub1 in yeast (3).

As for PML (19), SUMO-1-modified p73 may have a particular subcellular localization because we have found that SUMO-1-modified p73 is preferentially isolated in the detergent insoluble fraction. We attempted to exclude the possibility that this result reflects preferential SUMO-1 cleavage in the soluble fraction during cell lysis by addition of protease inhibitor mixtures and by the detection of SUMO-1-modified RanGAP1 in the soluble fraction. However, RanGAP1 and PML have recently been reported to be differentially sensitive in vivo to the nuclear SUMO-1 hydrolase SENP1 (53). We cannot thus completely rule out a differential isopeptidase sensitivity of p73-SUMO-1 in the soluble and insoluble fractions as an explanation for our results, although the inclusion in cell washing and lysis buffers of N-ethylmaleimide, which has been shown to inhibit SUMO-1 hydrolases in vitro (47), did not increase the amount of SUMO-1-modified p73 in the soluble fraction.

We have so far been unable to identify p73 accumulation in PODs that contain a large amount of nuclear SUMO-1 after MG132 treatment. However, the low percentage of p73 that is modified by SUMO-1 makes identification of this fraction uncertain. For p53, nuclear aggregates induced by leptomycin B treatment (which prevents nuclear export), have recently been localized adjacent to PODs (54).

Is SUMO-1 Modification Involved in the Transcriptional Activity of p73α?—Two recent reports (23, 24) show that SUMO-1 modification of p53 on lysine 386 increases the transactivation activity of p53 on reporter genes. We did not find this result examining activation by p73α, or by p53, of the RGC p53-responsive element in SK-NS cells. This may reflect experimental differences in promoter constructs, in cell types, or in levels of SUMO-1 modification.

We find here, as previously reported (46, 56), that the β isoform, which is not subject to SUMO-1 modification, is more transcriptionally active than the α isoform, which can be SUMO-1-modified. The C-terminal region of p73α has been shown to modulate its transcriptional and growth regulatory properties (55–57), acting both as a positive and negative regulator. Although our experiments do not provide evidence for a direct effect of SUMO-1 modification on the transcriptional activity of transfected p73, such a modification could indirectly modulate activation of the endogenous p73α protein by influencing interaction with other co-regulatory proteins such as the c-Abl tyrosine kinase (27) or the histone deactetylation complex (see below).

Novel SUMO-1-interacting Proteins and a SUMO-1-interacting Motif—Among the proteins we originally isolated in our p73 two-hybrid screen, the majority were subsequently found to interact with SUMO-1. These include PML, PM-Scl 75, thymine DNA glycosylase, PIASx, PKY, CHD3/ZFH, and one of the SUMO-1-activating enzymes, SEA2. Five of the protein sequences interacting in the two-hybrid system with SUMO-1 contained a motif with a central SXS (or SXT) triplet preceded by predominantly hydrophobic amino acids and followed by predominantly acidic amino acids (Fig. 2A). We have confirmed that this motif can interact with SUMO-1 in the two-hybrid system. The serine/threonine and acidic residues essential for this interaction constitute a double CKII kinase site (S/TXXED/E) (58), and the interaction may thus be regulated by phosphorylation.

Screening DNA sequence data bases for other proteins containing the SXS motif identified RanBP2/Nup358. Although the fit to our consensus sequence is not ideal (KKPEDPS DDDVL), in that acidic amino acids are found at positions normally constituted by hydrophobic amino acids (Fig. 2A), this sequence maps to the minimal domain determined for RanGAP1-SUMO-1 binding; 2550–2837 (59). A number of other potential SUMO-1-interacting proteins have been identified. These include c-Myc, DNA repair proteins (XPG, XRCC1, and the Ku70 regulatory subunit of the DNA protein kinase), centromeric proteins (CENP-B), components of the origin of repli-
cation (ORC1 and ORC2), and viral proteins such as the cytomegalovirus IE2 protein. In the latter case, this protein has been recently shown to be SUMO-1-conjugated (60).

The SXS motif has been functionally implicated in SUMO-1 interaction using the yeast two-hybrid system, where it may be interacting directly with SUMO-1 or indirectly via Ubc9 or one of the SUMO-1-activating enzymes. Recent experiments on the mouse homologue of PKP-related kinase PKM (61), the homeodomain-interacting protein kinase 2 (62) (Fig. 2A), show that the SXS motif is part of a sequence that specifies localization of mouse homeodomain-interacting protein kinase 2 in nuclear speckles and that interacts in vitro with Ubc9 (63). The SXS motif is also conserved in the PIAS transcription factor family (Fig. 2A), including the androgen receptor-interacting protein ARIP (64) and the protein Miz-1, a N-terminally truncated form of PIASxβ that interacts with the homeobox domain protein Mxs2 (65). These and other (66) findings suggest that global modulation of SUMO-1 levels might co-ordinately regulate transcription of diverse genetic programs.

SUMO-1-interacting Proteins and Transcriptional Repression—Another of the SUMO-1-interacting proteins is the CHD3/ZFH zinc finger-containing helicase, whose expression is associated with cell growth (39, 40). This protein does not contain an SXS motif but may be modified by SUMO-1 since it contains a potential SUMO-1 modification site (VKKE) within the 140 C-terminal amino acids identified here as the SUMO-1-interacting region. CHD3 has been shown to be present in histone deacetylase complexes (HDAC) (67), which have been implicated in transcriptional repression by p53 (68). This interaction between p53 and HDAC is indirect and is mediated at least in part by the co-repressor Sin3a. In the case of the lymphoid lineage-determining factors of the Ikaros gene family, interactions with HDAC have been shown to proceed both through Sin3 and through NURD/Mi-2 complexes (69, 70), the latter containing CHD3. In view of the interactions detected here, p73-CHD3 and p53-CHD3 interactions via SUMO-1 may also participate in transcriptional repression mediated by these proteins.

Other proteins interacting with both SUMO-1 and HDAC complexes include the homeodomain-interacting protein kinase 2 (62, 71) and unliganded nuclear receptors such as the androgen receptor and the glucocorticoid receptor (72–74). This may also be the case for retinoic acid receptors that were found to interact in two-hybrid studies with thymine DNA glycosylase (75), which we show here to interact with SUMO-1. The Drosophila transcriptional repressor Tramtrack 69 is also modified by SUMO-1 (76). SUMO-1 modification may play a key role in the balance between transcriptional activation and repression.

If this is true for p73 and its homologue p63, this may offer one explanation for the finding of N-terminally truncated forms of the α-isofroms of p63 and p73 (28, 77), which would be unable to activate transcription of target genes. These N-terminally truncated forms are, however, still able to bind to DNA, and could thus mediate transcriptional repression via SUMO-1-modulated interaction with HDAC complexes. Transcriptional repression by p53 is involved in the apoptotic activity of this protein (68), and this could also be the case for the apoptotic activity of p73 (24, 25), implicating both full-length and N-terminally truncated forms. Alternatively, N-terminally truncated forms can act as dominant negative inhibitors of p53- or p73-induced transcription and apoptosis (28, 48, 78, 79). The role of the different p73 forms in modulating apoptosis during development (80) and the contribution of SUMO-1 modification remain to be fully investigated.
SUMO-1 Modification of p73

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