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c-Jun and p53 Activity Is Modulated by SUMO-1 Modification*

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The ubiquitin-related SUMO-1 molecule has been shown recently to modify covalently a number of cellular proteins including IκBα. SUMO-1 modification was found to antagonize IκBα ubiquitination and protect it from degradation. Here we identify the transcription factors c-Jun and p53, two well known targets of ubiquitin, as new substrates for SUMO-1 both *in vitro* and *in vivo*. In contrast to ubiquitin, SUMO-1 preferentially targets a single lysine residue in c-Jun (Lys-229), and the abrogation of SUMO-1 modification does not compromise its ubiquitination. Activation of Jun NH_2-terminal kinases, which induces a reduction in c-Jun ubiquitination, similarly decreases SUMO-1 modification. Accordingly, loss of the two major Jun NH_2-terminal kinase phosphorylation sites in c-Jun, Ser-63 and Ser-73, greatly enhances conjugation by SUMO-1. A SUMO-1-deficient c-JunK229R mutant shows an increased transactivation potential on an AP-1-containing promoter compared with wild-type c-Jun, suggesting that SUMO-1 negatively regulates c-Jun activity. As with c-Jun, SUMO-1 modification of p53 is abrogated by phosphorylation but remains unaltered upon chemical damage to DNA or Mdm2-mediated ubiquitination. The SUMO-1 attachment site in p53 (Lys-386) resides within a region known to regulate the DNA binding activity of the protein. A p53 mutant, defective for SUMO-1 conjugation, shows unaltered ubiquitination but has a slightly impaired apoptotic activity, indicating that modification by SUMO-1 might be important for the full biological activity of p53. Taken together, these data provide a first link between the SUMO-1 conjugation pathway and the regulation of transcription factors.

Post-translational modifications with a variety of molecules, such as phosphate or acetate, play a crucial role in altering protein function. Ubiquitination represents a particular case where ubiquitin (Ub),[1] itself a small polypeptide, is linked to lysine residues in a protein to target it for proteasomal degradation (for review, see Ref. 1). Recently, several proteins that share similarity with Ub have been identified. One member of this ubiquitin-like protein family is SUMO-1, a polypeptide of 101 amino acids which can be attached covalently to proteins in a process that is mechanistically analogous to ubiquitination (for review, see Refs. 2 and 3). To date, the known substrates of SUMO-1 are RanGAP1 (4, 5), PML (6–8), Sp100 (8), and IκBα (9). The observation that RanGAP1 and PML are targeted to distinct subcellular structures upon conjugation to SUMO-1 suggested that modification by SUMO-1 might play an important role in regulating the subcellular localization of proteins. A strikingly different aspect of SUMO-1 modification was revealed more recently by Desterro *et al.* (9). They identified the NFκB inhibitor, IκBα, as a substrate for SUMO-1 and could show that SUMO-1 and Ub target the same lysine 21 in IκBα. Whereas ubiquitination of Lys-21 in IκBα requires phosphorylation of the adjacent Ser-32 and Ser-36 residues, SUMO-1 preferentially targets the stable, unphosphorylated form of IκBα, indicating that it acts antagonistically to Ub to protect IκBα from degradation.

Like IκBα, the transcription factors c-Jun and p53 are regulated by the Ub-proteasome pathway. c-Jun belongs to the AP-1 family of proteins whose members can form heterodimeric transcription complexes and are characterized structurally by the basic region-leucine zipper motif (for review, see Ref. 10). Mitogens or various forms of stress regulate either the transcriptional activity of c-Jun directly or the abundance of the protein by modulation of its stability. The p53 tumor suppressor acts mainly as a transcription factor on a number of genes whose products regulate cell cycle arrest and apoptosis (for review, see Ref. 11). p53 is subjected to multiple post-translational modifications, such as acetylation and phosphorylation, which regulate p53 stability, sequence-specific DNA binding, and biological activity (for review, see Refs. 12 and 13). In normal cells, p53 is kept labile by Mdm2, which promotes its degradation through the Ub-proteasome pathway (14, 15). Upon stress, such as DNA damage, the half-life of p53 is increased dramatically, leading to transcriptional activation of its target genes.

Intriguingly, both c-Jun and p53 have been reported to interact physically with the Ubc9 protein in a yeast two-hybrid assay (16, 17). Ubc9 is a SUMO-1-specific conjugating enzyme showing homology to E2-type Ub-conjugating enzymes. Ubc9 physically interacts with the known SUMO-1 substrates and seems to mediate the transfer of SUMO-1 to these proteins (2, 3). In this study, we show that c-Jun and p53 undergo SUMO-1 modification. The modification sites have been mapped, and the effect of SUMO-1 modification on the ubiquitination and activities of these proteins has been studied.

MATERIALS AND METHODS

Cell Culture, Transfection, and Flow Cytometry—HeLa and HT1299 cells were grown under standard conditions and were transfected using the LipofectAMINE Plus reagent (Life Technologies, Inc.) according to

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[1] The abbreviations used are: Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; HA, hemagglutinin; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; WCE, whole cell extract.

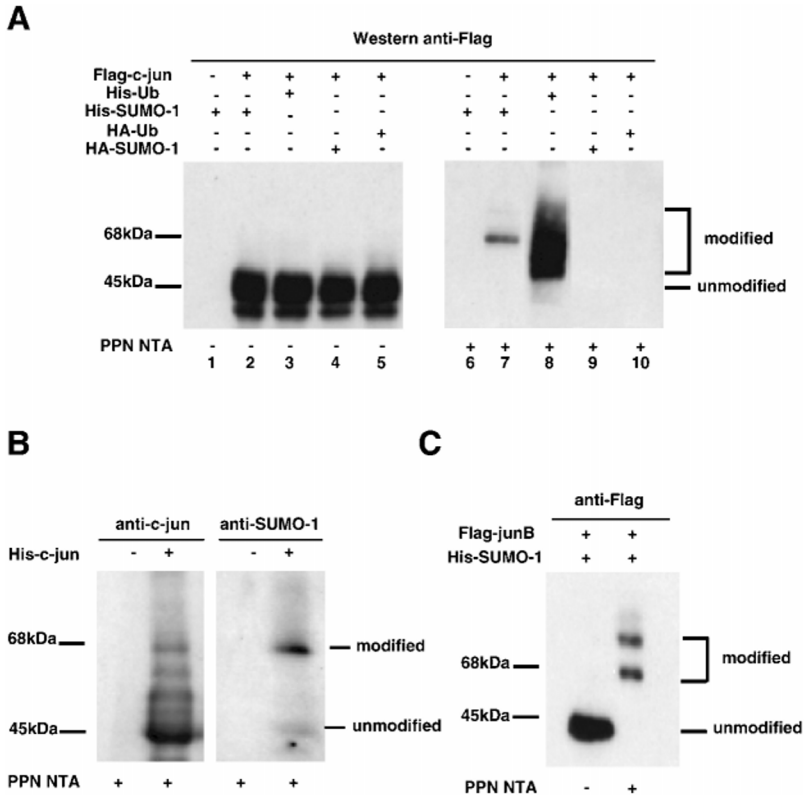

FIG. 1. **c-Jun and JunB are covalently modified by SUMO-1 *in vivo*.** *Panel A*, HeLa cells were transfected with the indicated plasmids. WCE (*lanes 1–5*) and NTA precipitates (*lanes 6–10*) were separated by SDS-PAGE and probed with an anti-Flag mAb. *Panel B*, NTA precipitates from HeLa cells transfected or not with His-c-Jun were examined by Western blotting with an anti-c-Jun (*left*) or an anti-SUMO-1 monoclonal antibody. *Panel C*, WCE and NTA precipitates from cells expressing Flag-JunB and His-SUMO-1 were analyzed by Western blotting with an anti-Flag monoclonal antibody. The positions of the unmodified c-Jun and its conjugates are indicated.

the manufacturer's protocol. Saos-2 cells were grown in RMPI and were transfected with calcium phosphate. Calyculin A (Sigma) was prepared as a 10 μM stock solution in dimethyl sulfoxide; the final concentration for cell treatment was 0.1 μM. For reporter assays, 3-cm Petri dishes were transfected with 1 μg of a reporter gene construct consisting of an adenovirus E3 promoter chloramphenicol acetyltransferase (CAT) fusion (18) and 200 ng of the indicated effector plasmids. CAT assays were performed as described (19), quantified by PhosphorImager analysis (ImageQuant) of thin-layer chromatograms, and normalized for transfection efficiency by using pCH110 and measuring β-galactosidase activity. The apoptotic assay was carried out by flow cytometry as described previously (20).

Antibodies, Plasmids, and Mutagenesis—The monoclonal anti-SUMO-1 antibody (19C7) was described previously (4). The monoclonal anti-Flag antibody (M2) was purchased from Sigma, the monoclonal anti c-Jun antibody (clone 3) from Transduction Laboratories, and the monoclonal anti-p53 antibody (Pab 1801) from Santa Cruz Biotechnology. The plasmid used for expression and *in vitro* translation of human p53 was pRC/CMV wild-type p53 (20). COOH-terminally Flag-tagged mouse c-Jun or JunB was cloned in pCG for mammalian expression or in pGEM (Promega) for *in vitro* translation (provided by D. Lallemand, L. Bakiri, and M. Yaniv). The His-tagged c-Jun expression vector as well as the vectors expressing hemagglutinin-tagged Ub (HA-Ub) and histidine-tagged Ub (His-Ub) were provided by M. Treier and D. Bohmann. NH_2-terminally His-tagged SUMO-1 was cloned in pSG5 (Stratagene). All site-directed mutagenesis were carried out using the quick change site-directed mutagenesis kit (Stratagene), and the mutant plasmids were subjected to DNA sequencing.

In Vitro Modification—The *in vitro* modification assay was carried out as described recently (9). *In vitro* translation reactions were done with the TNT-coupled reticulocyte lysate system (Promega).

Preparation of Cell Extracts, NTA Precipitation, and Western Blotting—36 h after transfection, cells were lysed in 1 ml of lysis buffer (6 M guanidinium HCl, 100 mM NaH_2PO_4, and 10 mM Tris-HCl (pH 7.8)). After sonication, 90% of the lysate was incubated with 25 μl of Ni-NTA magnetic agarose beads (Quiagen). The beads were washed twice with washing buffer (pH 7.8) containing 8 M urea, followed by washing with a buffer (pH 6.3) containing 8 M urea. After a final wash with phosphate-buffered saline, the beads were treated in SDS sample buffer for SDS-PAGE. 1/10th of the lysate was subjected to trichloroacetic acid precipitation and used as a whole cell extract (WCE). The proteins were analyzed by Western blotting using the appropriate antibodies as described recently (7).

RESULTS

c-Jun Is Modified Covalently by SUMO-1 in Vivo—Based on the observation that Ubc9 binds to c-Jun in a yeast two-hybrid assay, we hypothesized that c-Jun could be a target for SUMO-1 modification. To test this hypothesis *in vivo*, we used an assay initially developed by Treier *et al.* (21) to detect ubiquitination of c-Jun. In this assay, coexpression of c-Jun with His-Ub allows the purification of the ubiquitinated c-Jun forms by chromatography on nickel-charged agarose beads (NTA beads). In the modified system, His-Ub is replaced by a vector expressing a His-tagged SUMO-1 (His-SUMO-1) protein that, by analogy, should allow the NTA precipitation of putative c-Jun-SUMO-1 conjugates. HeLa cells were cotransfected with a vector expressing either His-SUMO-1 or His-Ub together with a vector expressing a Flag-tagged mouse c-Jun (Flag-c-Jun) protein. Both the unprecipitated WCE and NTA precipitates were analyzed by Western blotting with an anti-Flag monoclonal antibody. In crude extracts, a prominent 48-kDa band, corresponding to the unmodified c-Jun form, was detected (Fig. 1*A*, *lanes 2–5*). As observed previously in the NTA precipitates from cells expressing Flag-c-Jun together with His-Ub, a smear of bands representing the ubiquitinated forms of c-Jun was detected due to the enrichment of these forms on the NTA beads (Fig. 1*A*, *lane 8*). Strikingly, in the precipitates from cells expressing His-SUMO-1 together with Flag-c-Jun, a major anti-Flag reactive band migrating at 65 kDa was detected (Fig. 1*A*, *lane 7*). This band was not seen in cells expressing His-SUMO-1 alone (Fig. 1*A*, *lane 6*) demonstrating that it corresponds to a SUMO-1-modified form of c-Jun. After longer exposure, this c-Jun-SUMO-1 conjugate could also be detected as a very faint anti-Flag reactive band in the corresponding WCE (data not shown). The difference in the eletrophoretic mobility between the unmodified 48-kDa form and the 65-kDa c-Jun-SUMO-1 conjugate is consistent with the covalent attachment of one SUMO-1 molecule/molecule of c-Jun. To exclude any artifact that might be caused by unspecific

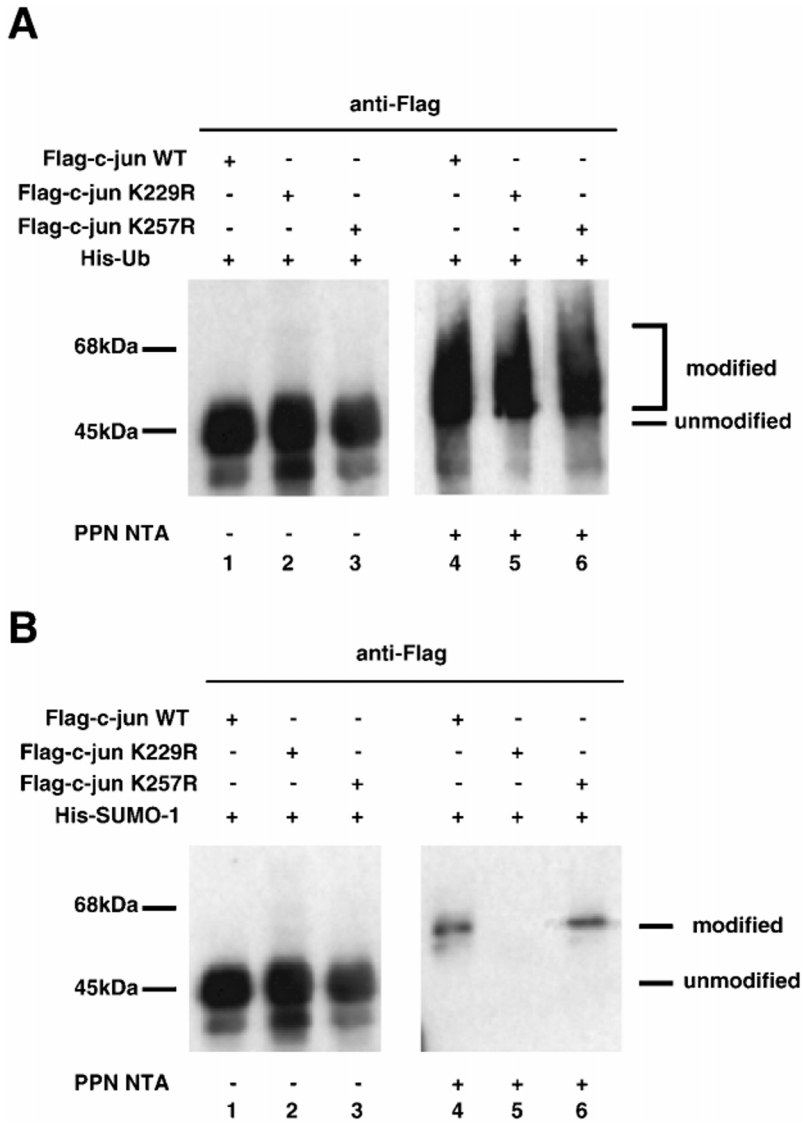

FIG. 2. **Lysine 229 is the major SUMO-1 modification site in c-Jun.** *Panel A*, wild-type Flag-c-Jun and the indicated Flag-c-Jun mutants were coexpressed with His-Ub. WCE (*lanes 1–3*) and NTA precipitates (*lanes 4–6*) were analyzed by Western blotting with the anti-Flag mAb. *Panel B*, wild-type Flag-c-Jun and the indicated Flag-c-Jun mutants were coexpressed with His-SUMO-1 and samples analyzed as in *panel A*.

binding of proteins to the agarose beads, we performed an analogous experiment replacing His-SUMO-1 or His-Ub by an HA-tagged version of SUMO-1 (HA-SUMO-1) or Ub (HA-Ub). Using this combination of vectors, c-Jun-Flag was detected in WCE (Fig. 1*A*, *lanes 4* and *5*), but, because of the absence of the His tag, no c-Jun-Ub or c-Jun-SUMO-1 conjugates were retained on NTA beads (Fig. 1*A*, *lanes 9* and *10*). It is noteworthy that overexpression of SUMO-1 in this assay does not induce a general nonspecific SUMO-1 modification of proteins that are otherwise ubiquitinated. For example, the well characterized ubiquitin substrate β-catenin did not become SUMO-1-modified under these conditions (data not shown).

To see whether c-Jun can be modified by endogenous SUMO-1, we transfected HeLa cells with a vector expressing a His-tagged c-Jun. NTA precipitates were separated by SDS-PAGE and probed with either a monoclonal anti-c-Jun antibody (Fig. 1*B*, *left panel*) or a monoclonal anti-SUMO-1 antibody (Fig. 1*B*, *right panel*). The anti-c-Jun antibody detected the major 48-kDa unmodified c-Jun protein as well as a series of protein bands ranging from 50 to 65 kDa. The slowly migrating 65-kDa band was strongly anti-SUMO-1 reactive, indicating that c-Jun is a substrate for endogenous SUMO-1.

To determine whether other members of the Jun family can undergo SUMO-1 modification, we tested JunB in the *in vivo* conjugation assay as described above by coexpressing a Flag-tagged JunB with His-SUMO-1. Similar to what observed for c-Jun, a SUMO-1-JunB conjugate migrating about 15 kDa above the 45-kDa unmodified form was retained on NTA beads (Fig. 1*C*). In addition, a second anti-Flag-reactive band at 75 kDa could be detected in the NTA precipitates. These two bands were detected equally after reprobing the membrane with an anti-SUMO-1 antibody (data not shown), indicating that two SUMO-1 molecules are linked covalently to JunB *in vivo*.

Lysine 229 Is the Major SUMO-1 Modification Site in c-Jun—With respect to the observed competition of SUMO-1 and Ub on a specific lysine residue of IκBα, we wished to identify the lysine(s) serving as the SUMO-1 attachment site(s) in c-Jun. Recently a short consensus sequence consisting of the L/I**K**X**E** motif has been identified as the SUMO-1 acceptor site in the known SUMO-1 substrates RanGAP1, PML, Sp100, and IκBα (22). Among the 18 lysines in the mouse c-Jun protein, we concentrated on lysines 229 (L**K**EE) and 257 (I**K**AE) because they reside in a region that matches this consensus motif. Lysines 229 and 257 were mutated independently to arginine by site-directed mutagenesis, and the ability of the mutants to be modified by Ub or SUMO-1 was tested in the NTA precipitation assay. Ubiquitination of either the K229R or the K257R mutant was equal to that of the wild-type c-Jun (Fig. 2*A*, *lanes 4–6*). This is consistent with the observation of Treier *et al.* (21)

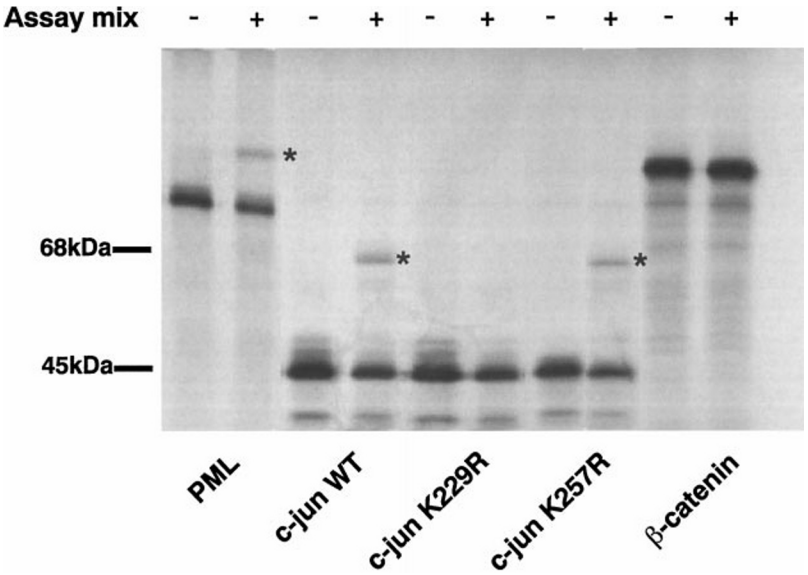

FIG. 3. **SUMO-1 modification of c-Jun can be reconstituted *in vitro*.** The indicated proteins were *in vitro* translated and incubated either in the absence (−) or presence (+) of the assay mix containing SUMO-1, Ubc9, and a fraction from HeLa cells providing the E1 activity. SUMO-1 conjugates are marked by an *asterisk*.

that mutation of Lys-226 or Lys-254 in human c-Jun, which correspond to Lys-229 and Lys-257 in mouse c-Jun, does not alter the ubiquitination of the human c-Jun protein. By contrast, Lys-229 mutation abrogated c-Jun modification by SUMO-1, whereas Lys-257 mutation had no effect (Fig. 2*B*, *lanes 4* and *6*). These findings identify Lys-229 as the major SUMO-1 attachment site in c-Jun and indicate that loss of SUMO-1 modification does not significantly alter the ubiquitination of c-Jun.

SUMO-1 Modification of c-Jun Can Be Reconstituted in Vitro—After having established the SUMO-1 modification of c-Jun *in vivo*, we wished to see whether we could reconstitute this modification in an *in vitro* system recently described by Desterro *et al.* (9). In this system, ^{35}S-labeled c-Jun generated by *in vitro* translation is used as a substrate in the presence of recombinant SUMO-1, recombinant Ubc9, and a fraction from HeLa cells providing the SUMO-1-activating E1 activity. The modification by SUMO-1 of the PML protein was used as a positive control (22). As can be seen in Fig. 3, the addition of E1, Ubc9, and SUMO-1 to the *in vitro* translated 75-kDa PML protein induces the formation of one major SUMO-1-PML conjugate visible by the appearance of a new 90-kDa PML form. Accordingly, the addition of the assay mix to *in vitro* translated wild-type c-Jun or K257R mutant induces the formation of a 65-kDa c-Jun-SUMO-1 conjugate. Consistent with the *in vivo* data, the c-Jun K229R mutant is no longer able to undergo SUMO-1 modification. The β-catenin protein, used as a negative control, remained unmodified in this system, demonstrating the specificity of this *in vitro* assay. Overall these data provide further evidence that c-Jun is a natural substrate for SUMO-1 modification on a specific lysine residue.

Heat Shock Down-regulates SUMO-1 Modification of c-Jun, whereas Loss of the NH_2-terminal Phosphorylation Sites Favors SUMO-1 Modification—Stress signals, such as UV irradiation or heat shock, trigger the stabilization of the c-Jun protein by protecting it from ubiquitination and subsequent degradation (for review, see Ref. 23). To see whether SUMO-1 modification of c-Jun is altered upon heat shock, HeLa cells were cotransfected with expression vectors encoding either His-SUMO-1 or His-Ub together with the Flag-c-Jun construct and subjected to heat shock before preparation of the extracts and NTA precipitation. The results are shown in Fig. 4*A*. As can be seen in WCE, c-Jun is expressed equally in control cells and in cells that had been exposed to heat shock (compare *lane 2* with *1* and *lane 6* with *5*). In agreement with published results, the amount of ubiquitinated c-Jun in the NTA precipitates was reduced after heat shock (compare *lane 4* with *3*). Strikingly, heat shock triggered a similar down-regulation of SUMO-1 modification as demonstrated by the lower amount of c-Jun-SUMO-1 conjugates retained on the NTA beads compared with untreated controls (compare *lane 8* with *7*).

Because ubiquitination of c-Jun is down-regulated by NH_2-terminal phosphorylation of c-Jun at serines 63 and 73 after stress-induced activation of JNKs, we assessed the role of these residues in the regulation of SUMO-1 modification (24). Both serines were changed to alanines, and SUMO-1 modification of this constitutively nonphosphorylated S63A,S73A mutant was compared with that of the wild-type protein in the *in vivo* modification assay. As can be seen in WCE, the two proteins were expressed at a similar level (Fig. 4*B*, *lanes 1*, *2*, *5*, and *6*). Consistent with previous findings (25, 26), mutation of the two serines resulted in a sharp increase in c-Jun ubiquitination (Fig. 4*B*, compare *lane 4* with *3*). Remarkably, the S63A,S73A mutant of c-Jun was modified more efficiently by SUMO-1 than the wild-type c-Jun protein (Fig. 4*B*, compare *lane 8* with *7*), and the 65-kDa SUMO-1-modified species could even be seen in crude extracts in the absence of NTA precipitates (Fig. 4*B*, *lane 6*). Taken together, these results indicate that SUMO-1 modification of c-Jun is, like ubiquitination, down-regulated by stress signals, presumably because of the JNK-mediated phosphorylation of residues Ser-63 and Ser-73.

SUMO-1 Modification Negatively Regulates the Transcriptional Activity of c-Jun—To get further insight into the functional role of SUMO-1 modification on c-Jun, we studied the impact of SUMO-1 modification on the transactivation potential of c-Jun. To this aim, we performed reporter gene assays on an adenovirus early region 3 (E3) promoter CAT reporter construct that harbors an AP-1 site (27). HeLa cells were cotransfected with the reporter together with empty vector, wild-type c-Jun, the SUMO-1-deficient c-JunK229R mutant or the phosphorylation deficient c-Jun S63A,S73A mutant. The results are summarized in Fig. 5; the data represent the mean (±S.E.) of five independent transfections. Whereas wild-type c-Jun activates transcription 3.1-fold (±0.07) compared with empty vector, the promoter activity was stimulated 4.2-fold (±0.19) by the SUMO-1-deficient c-JunK229R, representing an increase of about 35% compared with wild-type c-Jun. By contrast, loss of the NH_2-terminal phosphorylation sites Ser-63 and Ser-73 impairs the transcriptional activity by about 35% (2.0-fold, ±0.11) compared with wild-type c-Jun, which is consistent with the

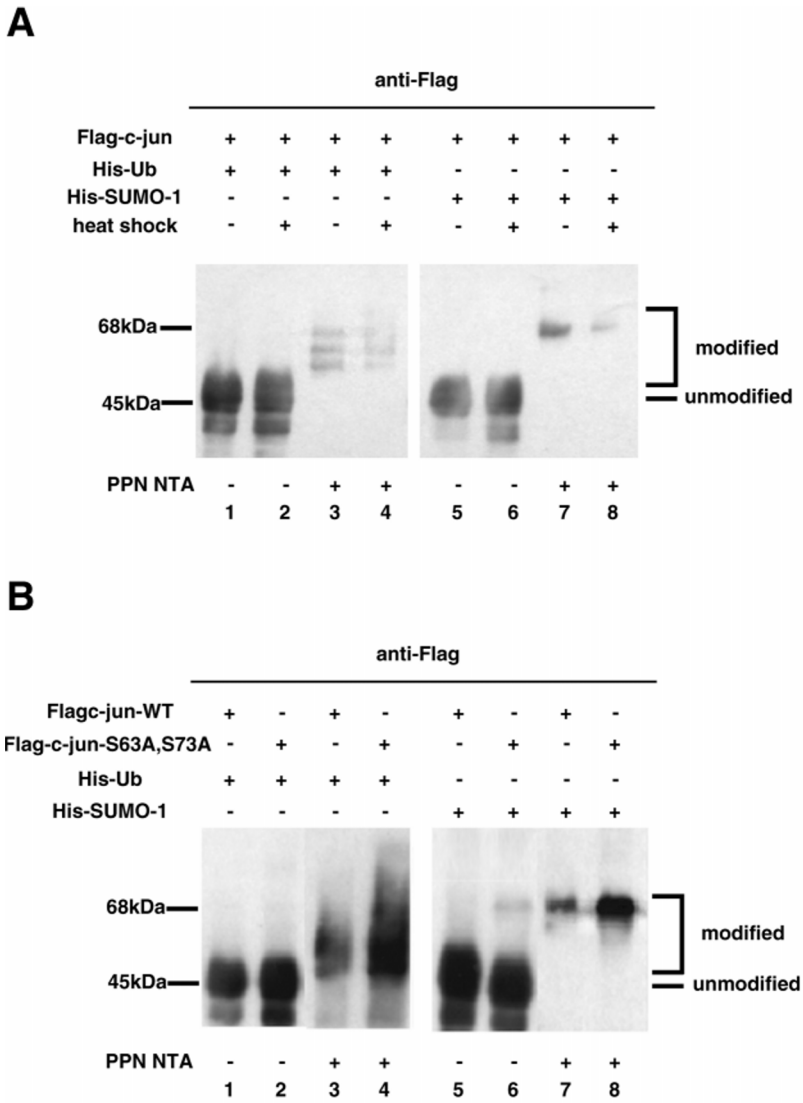

FIG. 4. **Heat shock down-regulates SUMO-1 modification of c-Jun, whereas loss of the NH_2-terminal phosphorylation sites favors SUMO-1 modification.** *Panel A*, HeLa cells were transfected with the indicated plasmids, and 36 h after transfection the cells were either untreated or exposed to a 42 °C heat shock for 45 min before preparation of the extracts and NTA precipitation. WCE and NTA precipitates were analyzed by Western blotting with an anti-Flag mAb. *Panel B*, cells were transfected with the indicated plasmids and extracts, and NTA precipitates were analyzed as in *panel A*.

established role of NH_2-terminal phosphorylation in c-Jun activation. All c-Jun proteins were expressed at equal levels as judged by immunoblotting (data not shown). Taken together, these data indicate that SUMO-1 modification negatively regulates the transcriptional activity of c-Jun.

p53 Is Modified by SUMO-1 at Residue 386 in Vivo and in Vitro—Similar to what has been described for c-Jun, the p53 protein has been reported to interact with Ubc9 in a yeast two-hybrid assay (16). This prompted us to test p53 for its capacity to undergo SUMO-1 modification. The ubiquitination was monitored in parallel. To this purpose, HeLa cells were cotransfected with human p53 together with either His-SUMO-1 or His-Ub and both WCE and NTA precipitates were analyzed by Western blotting using an anti-p53 monoclonal antibody. In HeLa cells, exogenously expressed p53 is ubiquitinated efficiently because of the presence of the papillomavirus E6 and the cellular E3 ligase E6AP. In WCE from cells expressing p53, the protein was detected as a major 50-kDa band (Fig. 6*A*, *lanes 1–4*). After purification on NTA beads, a ladder of ubiquitinated p53 forms ranging from 60 to 90 kDa was detected in extracts coexpressing His-Ub and p53 (Fig. 6*A*, *lane 6*). Remarkably, a p53-SUMO-1 conjugate migrating at 65 kDa was recovered on NTA beads from extracts expressing His-SUMO-1 together with p53 (Fig. 6*A*, *lane 5*), indicating that SUMO-1 can be covalently attached to p53 *in vivo*. When high transfection efficiency was achieved, the 65-kDa p53-SUMO-1 conjugate was readily detectable in WCE (see Fig. 6*B*, *lane 1*). When the HA tag was substituted for the His tag (Fig. 6*A*, *lanes 7* and *8*), although a residual binding of the unmodified p53 form was visible, the modified p53 species were not retained on the beads thus demonstrating the specificity of the conjugates.

Among the 20 lysines in human p53, lysine 386 (F**K**TE) resides in a region closely matching the consensus motif for SUMO-1 modification. This residue was thus mutated to arginine and the mutant tested for its capacity to be SUMO-1-modified *in vivo*. The expression level of K386R was similar to that of the wild-type p53 protein (Fig. 6*B*, *lanes 1–4*). Although ubiquitination of K386R was unaltered compared with that of the wild-type protein (Fig. 6*B*, compare *lane 8* with *6*) the mutant protein could no longer undergo SUMO-1 modification as demonstrated by the absence of the p53-SUMO-1 conjugate in both WCE and NTA precipitates (Fig. 6*B*, compare *lane 3* with *1* and *lane 7* with *5*).

To confirm further the absence of SUMO-1 modification of K386R, we used the more sensitive *in vitro* assay as described above. Incubation of *in vitro* translated p53 with the assay mix containing SUMO-1, Ubc9, and the E1 activity triggered the formation of a 65-kDa p53-SUMO-1 conjugate (Fig. 6*C*), demonstrating that p53 is modified efficiently *in vitro* by the attachment of a single molecule of SUMO-1. Consistent with the results obtained *in vivo*, the K386R mutant was completely deficient in SUMO-1 modification. Altogether, these experi-

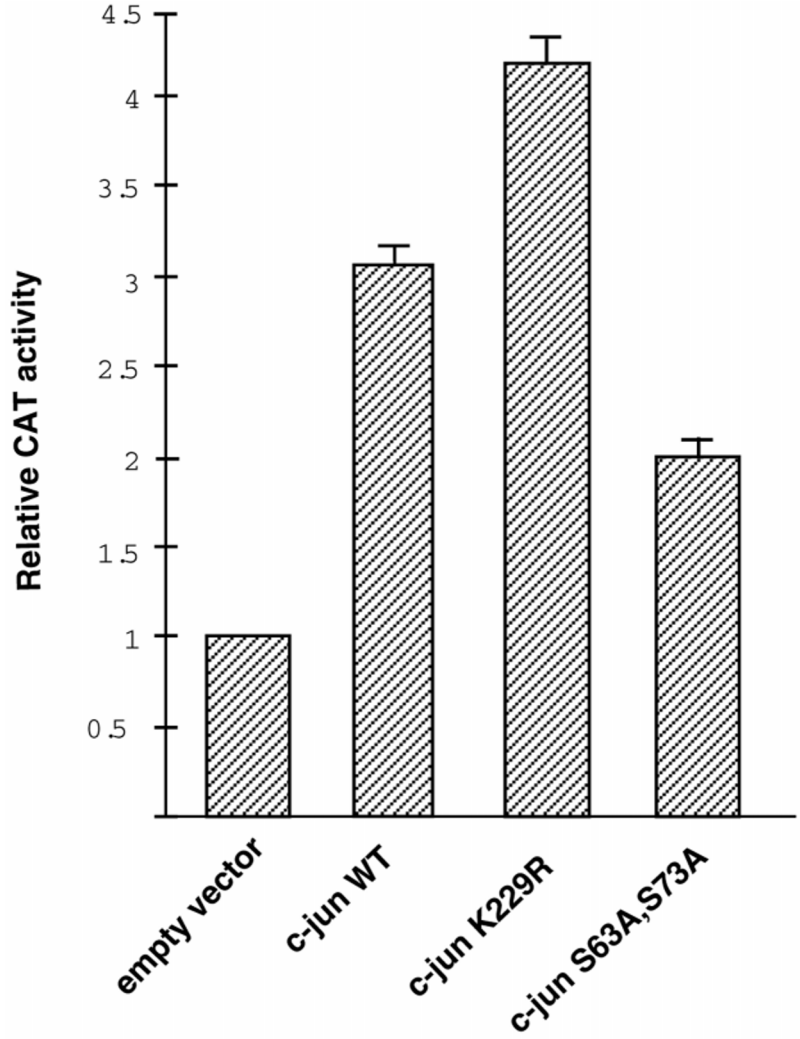

FIG. 5. **SUMO-1 modification negatively regulates the transcriptional activity of c-Jun.** HeLa cells were transiently transfected with the adenovirus early region 3-CAT reporter plasmid (1 μg) together with pCH110 (expressing β-galactosidase) and 200 ng of the effector plasmids as indicated. 24 h after transfection CAT activity was measured. Values represent the average of five independent transfections (±S.E.) after normalization for the internal control β-galactosidase activity of pCH110. CAT activities are expressed relative to basal promoter activity, which was set at 1.

ments show that lysine 386 is required for SUMO-1 modification of p53 *in vivo* and *in vitro* and that this residue is unlikely to serve as a major ubiquitination site in p53.

SUMO-1 Modification of p53 Is Unaltered upon Mdm2 Expression or DNA Damage but Is Abrogated by Phosphorylation—With regard to a possible role of SUMO-1 in counteracting ubiquitination, we wished to examine whether SUMO-1 modification of p53 was altered upon induction of p53 ubiquitination by Mdm2. To this aim, p53-negative H1299 cells were cotransfected with a p53 expression vector in combination with His-Ub, His-SUMO-1, and/or Mdm2 expression plasmids (Fig. 7*A*). Both the NTA precipitates and the WCE were analyzed by Western blotting using an anti-p53 antibody. Although the steady-state level of p53 ubiquitination in H1299 cells was relatively low (Fig. 7*A*, *lane 6*), a dramatic increase was noted upon expression of Mdm2 (Fig. 7*A*, *lane 8*), which is consistent with the function of Mdm2 as an E3 Ub ligase (28). By contrast, the amount of p53-SUMO-1 conjugates was unaffected by Mdm2 expression (Fig. 7*A*, compare *lane 5* with *7*), indicating that the induced ubiquitination is not associated with reduced SUMO-1 modification. In addition, exposure of H1299 cells to DNA damage by adriamycin or actinomycin D did not affect the extent of SUMO-1 modification, although it reduced p53 ubiquitination and elevated its stability (data not shown). Overall, these data argue against a role of SUMO-1 in regulating the ubiquitination of p53.

Because the ubiquitination of p53 can be regulated by phosphorylation, we wished to investigate whether SUMO-1 modification was subjected to a similar type of regulation. HeLa cells cotransfected with either His-SUMO-1 or His-Ub and p53 vectors were treated with calyculin A, a potent inhibitor of serine/threonine phosphatases 1 and 2A. As can be seen in Fig. 7*B*, calyculin A dramatically reduced the ability of p53 to be both ubiquitinated (compare *lane 8* with *6*) and SUMO-1-modified (compare *lane 7* with *5*). These data indicate that hyperphosphorylation prevents the formation of p53-SUMO-1 conjugates.

SUMO-1 Modification and Apoptotic Activity of p53—To study the possible effect of SUMO-1 modification on p53 biological activity, we compared the apoptotic potential of wild-type p53 with that of mutant K386R, which is deficient in SUMO-1 modification, using a transient transfection assay we described recently (29). In brief, Saos-2 cells (p53-negative) were transfected with either a vector expressing wild-type p53 or K386R; 72 h post-transfection, cells were collected, stained for p53, and analyzed by flow cytometry. The result of one representative experiment is shown in Fig. 8. Transfected and nontransfected cells can be distinguished easily by their level of fluorescent intensity (Fig. 8*A*). The cell cycle distribution of both the nontransfected (Fig. 8*B*) and transfected subpopulation (Fig. 8, *C* and *D*) of cells was analyzed separately, and cells with a DNA content of less than 2N (*Sub-G1* in Fig. 8*B*) were considered apoptotic. Wild-type p53 induces apoptotic cell death in 31% of cells (Fig. 8*C*), whereas the fraction of apoptotic cells is slightly reduced to 25% in cells expressing K386R (Fig. 8*D*). As shown in Fig. 8*E*, this difference cannot be attributed to

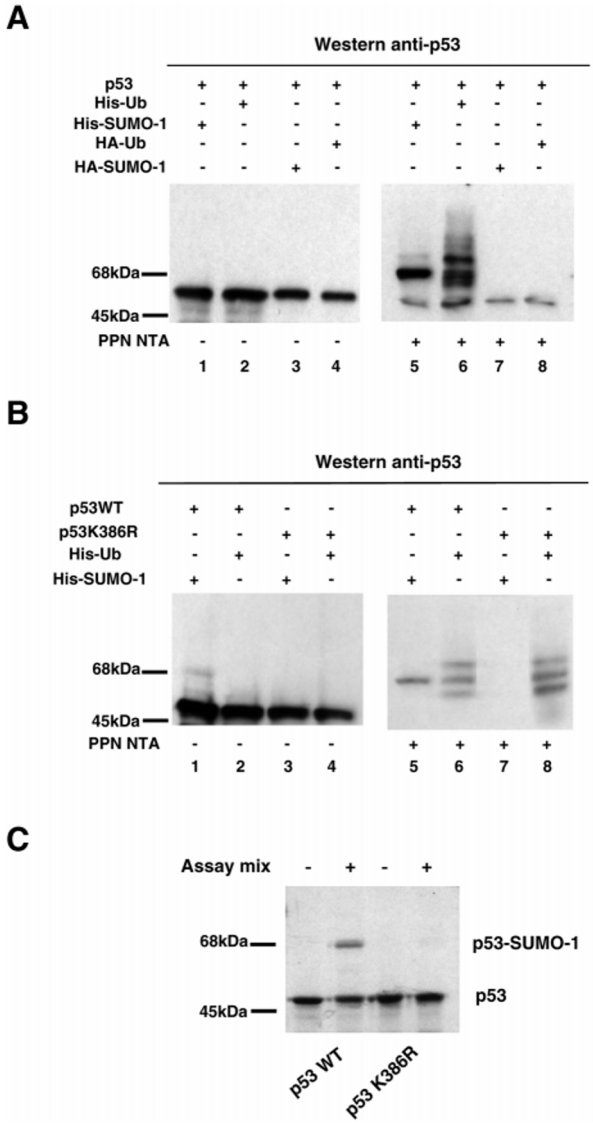

FIG. 6. **p53 is modified by SUMO-1 at residue 386 *in vivo* and *in vitro*.** *Panel A*, HeLa cells were transfected with the indicated plasmids. WCE (*lanes 1–4*) and NTA precipitates (*lanes 5–8*) were separated by SDS-PAGE and probed by Western blotting with an anti-p53 mAb. *Panel B*, HeLa cells were transfected with the indicated plasmids and analyzed as in *panel A*. *Panel C*, wild-type p53 and p53K386R were *in vitro* translated and submitted to the *in vitro* modification assay as described in Fig. 3.

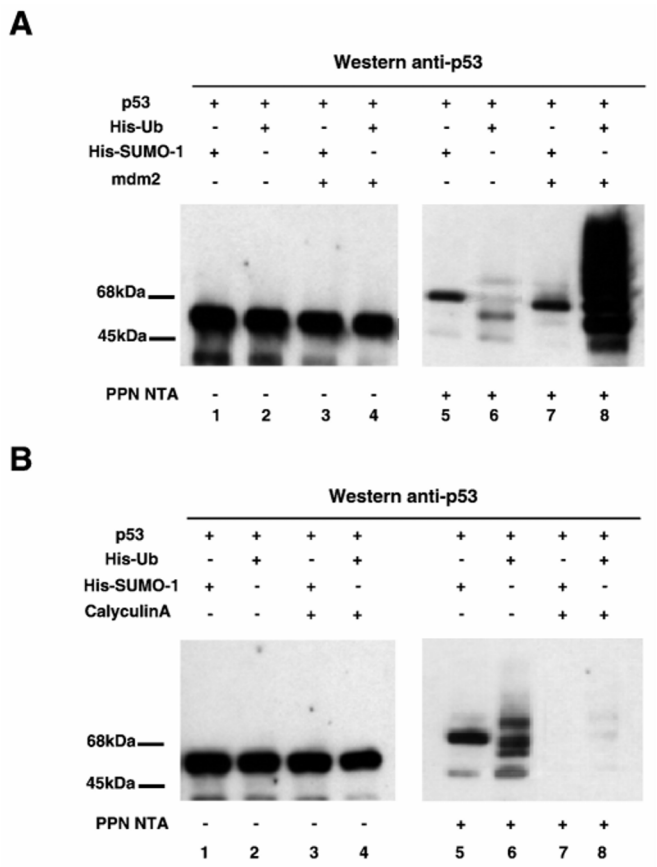

FIG. 7. **SUMO-1 modification of p53 is not affected by Mdm2 expression but is abrogated by phosphorylation.** *Panel A*, H1299 cells were transfected with the indicated plasmids. WCE (*lanes 1–4*) and NTA precipitates (*lanes 5–8*) were separated by SDS-PAGE and probed by Western blotting with an anti-p53 mAb. *Panel B*, HeLa cells were transfected with the indicated plasmids. Cells were either untreated or treated with 0.1 μM calyculin A for 45 min before cells lysis and NTA. Samples were analyzed as in *panel A*.

variations in the expression levels of wild-type p53 and K386R under the experimental conditions used. Although this difference was rather modest, it was consistent over a number of independent experiments. With cumulated data from seven samples (for each wild-type p53 and p53K386R) of three independent experiments we obtained a mean of 31.7% (S.E. $\pm$ 1.32) apoptotic cells for wild-type p53 and a mean of 25.4% (S.E. $\pm$ 0.3) of apoptotic cells for p53K386R. Thus, compared with the wild-type p53 protein, the apoptotic potential of K386 was impaired by about 20%, suggesting that SUMO-1 modification of p53 may be necessary for exerting its full apoptotic activity.

DISCUSSION

In this report, we show that the transcription factors c-Jun and p53 can be covalently modified by SUMO-1 both *in vitro* and *in vivo*. The activity of c-Jun and p53 is tightly regulated by post-translational modifications, and, similarly to what has been shown for IκBα, the interplay between phosphorylation and ubiquitination plays a pivotal role in the control of their stability. Intriguingly, Desterro *et al*. (9) could show that the unphosphorylated, stable IκBα protein is SUMO-1-modified, whereas upon phosphorylation, this residue becomes ubiquitinated to induce degradation of IκBα. Our data provide compelling evidence that this kind of antagonism between Ub and SUMO-1 is not seen on c-Jun. First, in contrast to Ub, SUMO-1 preferentially targets a single lysine in c-Jun, and mutation of this site does not alter ubiquitination. Second, for both Ub and SUMO-1, the unphosphorylated c-Jun is a better substrate than the phosphorylated form. Third, induction of Ser-63, Ser-73 phosphorylation by heat shock down-regulates both ubiquitination and SUMO-1 modification of c-Jun. Taken together, these data strongly argue against a role of SUMO-1 in protecting c-Jun from degradation. Consistent with this idea, the half-life of c-Jun was found to be unaltered upon loss of SUMO-1 modification in pulse-chase experiments.[2] The observed down-regulation of SUMO-1 modification upon c-Jun activation suggests that SUMO-1 has an inhibiting role on c-Jun activity. Accordingly, the SUMO-1-deficient c-JunK229R mutant is transcriptionally more active on an AP-1-containing promoter than the SUMO-1 modified wild-type c-Jun protein. The modulation of c-Jun activity by SUMO-1 linkage by about 35% is quantitatively similar to the well established regulatory effect of NH_2-terminal c-Jun phosphorylation, indicating that

[2] S. Müller and A. Dejean, unpublished results.

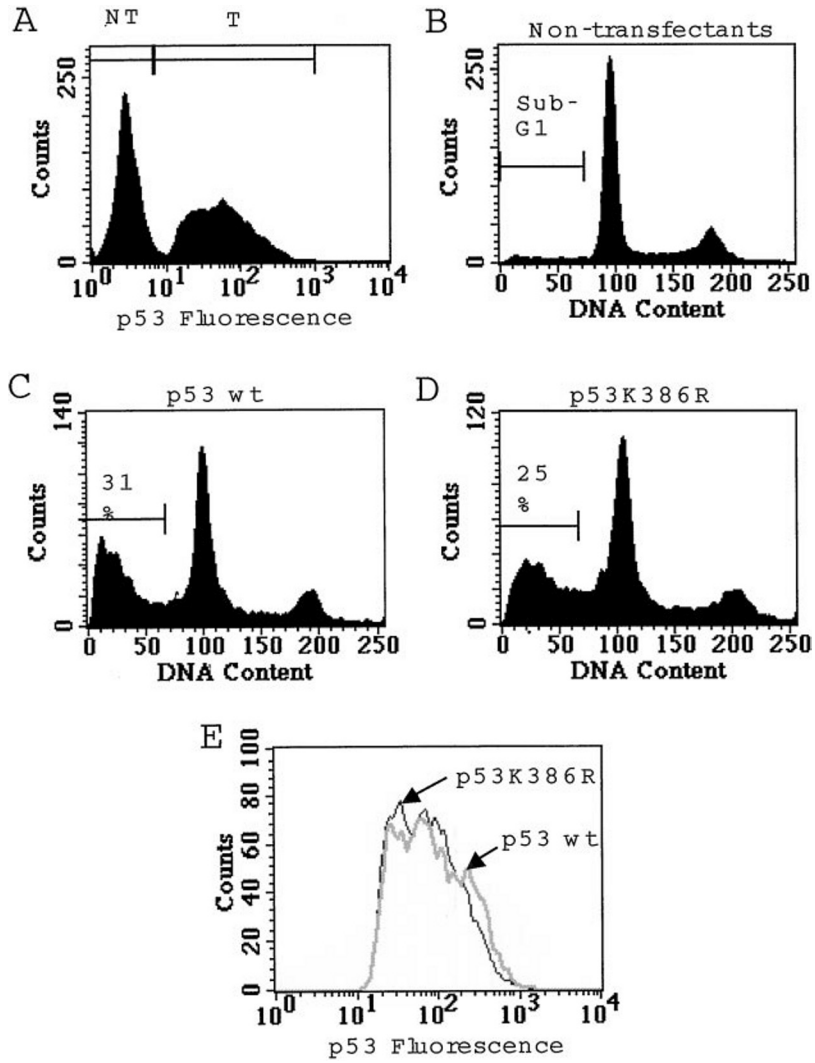

FIG. 8. **SUMO-1 modification and apoptotic activity of p53.** Saos-2 cells were transfected with either wild-type p53 or p53K386R. 72 h post-transfection, cells were harvested and stained for p53. Stained cells were then subjected to flow cytometric analysis. *Panel A*, levels of p53 fluorescence in the transfected culture; the nontransfected subpopulation (*NT*) and the transfected one (*T*) are indicated. Note that fluorescence is plotted on a logarithmic scale. *Panel B*, cell cycle distribution of the nontransfected population as determined by propidium iodide staining. The region of apoptotic cells is marked (*Sub-G1*). *Panel C*, DNA content distribution of cells transfected with wild-type p53 (subpopulation *T* of *panel A*); the percentage of apoptosis is indicated. *Panel D*, as in *panel C*, except cells were transfected with p53K386. *Panel E*, histogram showing the p53 fluorescence intensity distribution among cells transfected with wild-type p53 or with p53K386R.

similarly to phosphorylation, SUMO-1 modification plays an important role in regulating c-Jun activity. It remains to be determined whether SUMO-1 modification directly modulates the affinity of c-Jun for specific DNA binding or regulates its capacity to interact with transcriptional cofactors.

Similar to c-Jun, p53 undergoes distinct post-translational modifications at various sites. Subsequent to DNA damage, Ser-15 and/or Ser-20 are phosphorylated, which attenuates the p53/Mdm2 interaction and induces the accumulation of p53 (20, 30). In addition to causing p53 accumulation, post-translational modifications activate p53 as a transcription factor. Phosphorylation or acetylation of residues in the extreme COOH-terminal region seems to activate sequence specific DNA binding of p53. According to a recently proposed model (31, 32), the positively charged COOH-terminal tail interacts with the core DNA binding domain and locks it into an inactive conformation. Loss of basic charges upon acetylation would disrupt this interaction and allow the DNA binding domain to adopt an active conformation. Intriguingly, the SUMO-1 attachment site is localized in this COOH-terminal region and, like acetate, SUMO-1 neutralizes the basic charge of a lysine, raising the possibility that it may induce the conformational change in p53 necessary for its full activation. This is supported by the observation that the K386R mutant, which is no longer conjugated by SUMO-1, has a slightly impaired apoptotic potential. The rather slight difference in the biological activities of K386R mutant compared with wild-type p53 might reflect the fact that in transient transfections, the proportion of SUMO-1-modified p53 remains low. In addition to the impaired apoptotic potential, we observed a modest impairment in the transactivation potential of p53 on a p21 promoter upon replacement of Lys-386 by arginine (data not shown). In agreement with that, two very recent publications provided strong evidence that SUMO-1 stimulates the transcriptional activation of p53 (33, 34). To elucidate further the biological role of the SUMO-1 modification of p53, it will be necessary to determine the signals that regulate the equilibrium between the modified and unmodified form. Although no alteration was observed upon treatment of cells with the DNA-damaging agent adriamycin, other forms of genotoxic stress might be able to induce SUMO-1 modification as there is evidence that different types of DNA damage are relayed through discrete pathways to p53. Our observation that a phosphatase inhibitor triggers a decrease in SUMO-1 modification supports the idea that this process is regulated negatively by phosphorylation of p53 at one or more serine/threonine residues. Similar to what is noticed for c-Jun, our present data make it unlikely that SUMO-1 can compete directly with Ub on p53. In accordance to previous reports (35, 36), Lys-386 did not seem to serve as a major ubiquitination site in p53, and no alteration of SUMO-1 modification of p53 was seen in situations where the ubiquitination of p53 is either induced, following Mdm2 expression, or suppressed, following DNA damage.

Taken together, our data indicate that transcription factors

can undergo modification by SUMO-1, and, in analogy to ubiquitination or acetylation, SUMO-1 modification seems to be tightly regulated by phosphorylation. Our data provide evidence that conjugation by SUMO-1 modulates the biological activity of c-Jun and p53. Although the exact mechanism of how SUMO-1 regulates p53 and c-Jun activity remains to be elucidated, the identification of this modification adds further complexity to the post-translational regulation of these proteins and will open up new perspectives in their study.

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