Expression of SUMO-2/3 Induced Senescence through p53- and pRB-mediated Pathways*

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Three highly homologous small ubiquitin-related modifier (SUMO) proteins have been identified in mammals. Modifications of proteins by SUMO-1 have been shown to regulate transcription, nucleocytoplasmic transport, protein stability, and protein-protein interactions. Relative to SUMO-1, little is known about the functions of SUMO-2 or SUMO-3 (referred to as SUMO-2/3). Here, stable cell lines overexpressing processed forms of SUMO-2/3 (SUMO-2/3GG) as well as their non-conjugatable derivatives, SUMO-2/3ΔGG, were established. Cells overexpressing SUMO-2/3GG showed a premature senescence phenotype as revealed by cellular morphology and senescence-associated galactosidase activity. The senescence pathway protein p21 was up-regulated in cells overexpressing SUMO-2/3GG. In contrast, cells overexpressing non-conjugatable forms of SUMO-2/3ΔGG showed neither an apparent senescent phenotype nor elevated p21. Both p53 and pRB were found to be modified by SUMO-2/3. Site-directed mutagenesis studies showed that Lys-386 of p53, the SUMO-1 modification site, is also the modification site for SUMO-2/3. In addition, H2O2 treatment of untransfected cells caused an increase in p53 sumoylation by SUMO-2/3, whereas that by SUMO-1 remained unchanged. Moreover, knocking down tumor suppressor proteins p53 or pRB using small interfering RNA significantly alleviated the premature senescence phenotypes in SUMO-2/3GG overexpressing cells. Together, our results reveal that p53 and pRB can be sumoylated by SUMO-2/3 in vivo, and such modification of p53 and pRB may play roles in premature senescence and stress response.

The amino acid sequence of the small ubiquitin-modifier (SUMO)2 is only 18% identical to that of ubiquitin, but its three-dimensional structure is very similar to ubiquitin. SUMO is attached to its target proteins by an enzymatic mechanism that is analogous to the ubiquitinylation pathway (1–3). The mammalian SUMO family consists of SUMO-1, -2, and -3. Human SUMO-1 (SMT3C) exhibits 46% sequence identity with SUMO-2 (SMT3A) and SUMO-3 (SMT3B), whereas SUMO-2 and SUMO-3 are 96% identical in their processed forms; thus SUMO-2 and SUMO-3 are referred as SUMO-2/3 (4). SUMO-1 has been found to covalently modify >70 target proteins. Many of them are important regulatory proteins, such as p53, IκBα, c-Jun, promyelocytic leukemia protein (PML), and proliferating cell nuclear antigen (PCNA) (5, 6). The functions of SUMO-1 conjugation are target-specific and highly diverse. Sumoylation may be involved in the regulation of transcription, nucleocytoplasmic transport, DNA repair, protein stability, and chromosome separation (1, 2). Unlike SUMO-1, SUMO-2/3 can form polysumoylation chains due to their sequences containing the intrinsic SUMO consensus sequence ψKXE, where ψ stands for a large hydrophobic amino acid residue (7). Another important difference between SUMO-2/3 and SUMO-1 conjugation pathways in mammalian cells is that the majority of SUMO-1 exists in the conjugated forms, whereas SUMO-2/3 exist primarily as free forms and readily conjugate to substrates under certain stresses (8). In contrast to the large number of identified SUMO-1 target proteins, only a few SUMO-2/3-modified proteins have been reported. The physiological functions of SUMO-2/3 are not well understood.

Cellular premature senescence is a program triggered by cells in response to various types of stresses, including DNA damage, oxidative stress, and oncogene activation (9, 10). Cells entering senescence undergo permanent cell cycle arrest with a set of functional and morphological changes. The cellular signals activated by the stresses are funneled to p53 and pRB, which determine whether cells enter senescence (10, 11). Accordingly, most genes found to regulate senescence (i.e. p21 and p16INK4a) can be placed upstream or downstream of p53 or pRB in the signaling pathways (12). p53 can be modified with SUMO-1 at lysine 386, and the sumoylation of p53 enhances its transcriptional activity (13–15). Most recently, pRB was also found to be modified by SUMO-1, and its activity was regulated by sumoylation (16). Because p53 and pRB as well as SUMO-2/3 (but not SUMO-1) are involved in cellular stress responses, it is of interest to determine whether SUMO-2/3 can modify p53 and pRB as a possible regulatory mechanism in cellular senescence.

To further investigate the physiological functions of SUMO-2/3, we developed stable HEK293 Tet-On cell lines overexpressing Myc-His-SUMO-2/3GG and Myc-His-SUMO-2/3ΔGG. Our results reveal that p53 and pRB are modified by...
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SUMO-2/3, and premature cellular senescence also occurs in cells overexpressing SUMO-2/3. Furthermore, H$_2$O$_2$ induced p53 modification by SUMO-2/3 in untransfected HEK293 cells. These findings provide new insights into the potential regulatory role of sumoylation in cellular senescence in response to oxidative stress.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Mutagenesis—The monoclonal anti-Myc (9E10), anti-SUMO-1, anti-RanGAP1, anti-p53 (DO-1) antibodies, agarose-conjugated anti-Myc, and anti-p53 antibodies were purchased from Santa Cruz Biotechnology, monoclonal anti-HA and anti-β-actin antibodies from Sigma, monoclonal anti-p21 and anti-pRB antibodies from Upstate Biotechnology, and polyclonal anti-SUMO-2/3 antibody from Abcam. The cDNAs encoding the processed forms of SUMO-2/3 (1–97), SUMO-2 (1–90), and SUMO-3 (1–91) sequences, lacking the C-terminal Gly-Gly at their C termini were amplified by PCR. His$_6$ tag sequences immediately upstream of the start codons of the SUMO-1/2/3 sequences were designed in the amplifying primers. The PCR-amplified cDNAs were inserted into the pTRE2hyg2-Myc vector (Clontech) as Nhel/Clal fragments to generate pTRE2hyg2-Myc-His-SUMO-1/2/3GG plasmids. The pTRE2hyg2-Myc-His-SUMO-2/3ΔGG plasmids harboring non-conjugatable SUMO-2 (1–90) and SUMO-3 (1–91) sequences, lacking the C-terminal Gly-Gly, were similarly constructed. The wild-type p53 cDNA was amplified by PCR and inserted into the pTRE2pur-HA (Clontech) as a MluI/Clal fragment to generate the pTRE2pur-HA-p53WT plasmid. The pTRE2pur-HA-p53K386R (where Lys-386 is replaced by Arg) plasmid was constructed as described above using a 3′ primer specifically designed with the corresponding mutation. The p53 response reporter plasmids pRGC-Luc and pG13-Luc, were generous gifts from Dr. Seung J. Baek (University of Tennessee) and Dr. Ronald T. Hay (University of St. Andrews), respectively.

Cell Culture, Transfection, and Cell Lines—The stable HEK293 Tet-On or mouse embryonic fibroblast (MEF) 3T3 Tet-Off cell lines overexpressing Myc-His-SUMO-1/2/3GG and Myc-His-SUMO-2/3ΔGG were established using the protocol previously described (17). FuGENE6 (Roche Applied Science) was used for transient transfection.

Purification of His$_6$-tagged SUMO Substrates—The His$_6$-tagged proteins were purified under denaturing conditions using Ni-NTA-agarose beads in 2× NuPAGE sample buffer containing 250 mM imidazole. Proteins were resolved by 4–12% NuPAGE gels (Invitrogen) and probed with specific antibodies.

Immunoprecipitation and Immunoblotting—For immunoprecipitation, cells were lysed in lysis buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 20 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 20 μg/ml leupeptin, and 10 μg/ml aprotinin) to obtain whole cell extracts. Immunoprecipitation and immunoblotting were carried out as described previously (17).

Two-dimensional Gel Electrophoresis—The whole cell extracts from stable cell lines overexpressing SUMO-1/2/3GG were analyzed by two-dimensional gel electrophoresis as described previously (17).

p53 Transcriptional Transactivation Activity Assay—For the pRGc-Luc and pG13-Luc p53 response reporter plasmids, transfection was used to assess the transcriptional activity of p53 in vivo. The luciferase activity was determined with the Luciferase reporter assay kit (BioVision) using a Turner Design luminometer (Promega). The values obtained were normalized with protein concentration in each sample.

Senescence-associated Galactosidase Assays—The senescence-associated expression of β-galactosidase (SA-β-Gal) activity was monitored using a senescence detection kit (BioVision).

RNA Interference—The human p53 siRNA, human pRB siRNA, or control siRNA (Santa Cruz Biotechnology) was transfected into SUMO-2GG or SUMO-3GG stable cells using siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer’s instructions.

RESULTS

Enhancement of SUMO-2/3 Conjugations in Cells Expressing SUMO-2/3—Unlike SUMO-1, SUMO-2/3 are present mainly in their free forms instead of conjugated forms under normal conditions, and the conjugation of SUMO-2/3 increases significantly after certain stress conditions, such as heat shock and oxidative stress (8). Stable cell lines overexpressing the processed forms of SUMO-2/3 (Myc-His-SUMO-2/3GG) and their non-conjugatable mutants (Myc-His-SUMO-2/3ΔGG) were established to analyze the consequences of the increased levels of SUMO-2/3 conjugation during stresses. As shown in Fig. 1, Myc-His-SUMO-2/3GG (abbreviated as SUMO-2/3GG) and Myc-His-SUMO-2/3ΔGG (abbreviated as SUMO-2/3ΔGG) were successfully expressed in the respective cell lines. A large number of high molecular weight sumoylated proteins and unconjugated SUMO-2/3 was detected by probing with either anti-Myc or anti-SUMO-2/3 antibody in cells expressing SUMO-2/3GG. The expression level of SUMO-2GG was apparently higher than that of SUMO-3GG, correlating with the higher level of sumoylated proteins observed by anti-SUMO-2/3 antibody in SUMO-2GG cells (Fig. 1). However, only unconjugated SUMO-2/3 was detected by anti-Myc antibody, and the level of endogenous sumoylated high molecular weight proteins detected by anti-SUMO-2/3 antibody is relatively low in cells expressing SUMO-2/3ΔGG (Fig. 1). This observation indicates that epitope-tagged SUMO-2/3GG are
RanGAP1 was further confirmed by immunoprecipitation with anti-RanGAP1 antibody (Fig. 2A, lanes 3). The global patterns of SUMO target proteins by different SUMO paralogues were further characterized by two-dimensional gel electrophoresis. As shown in Fig. 2B, the overall sumoylation patterns between SUMO-2 and SUMO-3 are apparently similar, indicating that SUMO-2 and SUMO-3 may target the same proteins with similar intensity. In contrast, SUMO-1 modified many neutral proteins and fewer high molecular weight proteins. These results indicate that SUMO-1 and SUMO-2/3 have different substrate preferences that may function specifically under distinct conditions.

**Overexpressing Processed Forms of SUMO-2/3-induced Senescence**—Interestingly, cells overexpressing processed forms of SUMO-2/3 (SUMO-2/3GG) were found to grow slowly. Furthermore, many enlarged and flattened cells, a morphology commonly observed in senescent cells, were observed in the cells overexpressing SUMO-2/3GG (Fig. 3). Such a phenotype was not observed in cells overexpressing non-conjugatable forms of SUMO-2/3 (SUMO-2/3ΔGG). The SA-β-Gal activity was measured to confirm whether cells exhibiting the senescent morphology were indeed in senescence. As shown in Fig. 3, many senescent cells, stained blue due to SA-β-Gal activity, were found among cells overexpressing SUMO-2/3GG, but hardly any were found among cells overexpressing SUMO-2/3ΔGG. These results suggest that elevated cellular SUMO-2/3 modification levels in cells expressing SUMO-2/3GG may interfere with cell growth by inducing senescence. This unexpected observation with the immortal HEK293 cells was further confirmed in MEF 3T3 cells, a cell line typically used in senescence study. As shown in Fig. 3, overexpression of SUMO-2/3 also caused these cells to become enlarged and flattened, exhibiting SA-β-Gal activity.

**Knocking Down p53 or pRB Significantly Alleviated Senescence Phenotype**—The tumor suppressor proteins p53 and pRB are two major regulatory proteins known to mediate premature cellular senescence. We wondered whether knocking down these proteins would alleviate the effects of overexpressing SUMO-2/3GG-induced senescence. As shown in Fig. 4A, when cells overexpressing SUMO-2/3GG were transfected with either p53 siRNA or pRB siRNA for 48 h, cellular p53 or pRB protein level was reduced to about one-third of that observed in cells transfected with a control siRNA consisting of a non-targeting 20–25-nt RNA sequence. Furthermore, the senescence-associated β-galactosidase activity was significantly reduced in p53 or pRB knockdown cells (Fig. 4B). These results indicate that the SUMO-2/3-induced premature cellular senescence is dependent on both p53 and pRB pathways.

**p53 and pRB Are Modified by SUMO-2/3**—The His6-tagged sumoylated proteins from SUMO-2/3GG and SUMO-2/3ΔGG overexpressing cells were purified using Ni-NTA agarose and probed with anti-RanGAP1 antibody (Fig. 2A, lanes 3). The global patterns of SUMO target proteins by different SUMO paralogues were further characterized by two-dimensional gel electrophoresis. As shown in Fig. 2B, the overall sumoylation patterns between SUMO-2 and SUMO-3 are apparently similar, indicating that SUMO-2 and SUMO-3 may target the same proteins with similar intensity. In contrast, SUMO-1 modified many neutral proteins and fewer high molecular weight proteins. These results indicate that SUMO-1 and SUMO-2/3 have different substrate preferences that may function specifically under distinct conditions.

**Overexpression of processed and non-conjugatable forms of SUMO-2/3 in HEK293 Tet-On cells**. HEK293 Tet-On cells were transfected with pTRE2hyg2-Myc-His-SUMO-2/3GG or pTRE2hyg2-Myc-His-SUMO-2/3ΔGG plasmid as indicated. Cells stably expressing SUMO-2/3GG or SUMO-2/3ΔGG were selected with 300 μg/ml doxycycline, the whole cell extracts were resolved using NuPAGE gels and probed with anti-SUMO-1 or anti-SUMO-2/3 antibody, respectively. The sumoylation patterns of SUMO-1/2/3 in HEK293 cells. The sumoylation patterns of SUMO-1/2/3 in HEK293 cells—Similar but Different from That of SUMO-1—As shown in Fig. 2A, the majority of SUMO-1 is conjugated to the target proteins, whereas appreciable amounts of SUMO-2/3 are present in free forms. SUMO-1 modification apparently did not respond to H2O2 treatment; in contrast, SUMO-2/3 conjugates increased significantly after cells were treated with H2O2 (Fig. 2A, lanes 2). These results are consistent with a previous report (8). Furthermore, Western blot analysis using anti-SUMO-2/3 antibody revealed that RanGAP1, the most abundant SUMO-1 modified protein, was modified by SUMO-2/3. This sumoylation of RanGAP1 was further confirmed by immunoprecipitation with anti-RanGAP1 antibody (Fig. 2A, lanes 3). The global patterns of SUMO target proteins by different SUMO paralogues were further characterized by two-dimensional gel electrophoresis. As shown in Fig. 2B, the overall sumoylation patterns between SUMO-2 and SUMO-3 are apparently similar, indicating that SUMO-2 and SUMO-3 may target the same proteins with similar intensity. In contrast, SUMO-1 modified many neutral proteins and fewer high molecular weight proteins. These results indicate that SUMO-1 and SUMO-2/3 have different substrate preferences that may function specifically under distinct conditions.

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anti-RanGAP1, anti-p53, or anti-pRB antibodies, respectively. As shown in Fig. 5, protein bands corresponding to the sizes of the monosumoylated RanGAP1, p53, and pRB were observed in cells expressing SUMO-2/3GG but not in those expressing SUMO-2/3ΔGG. These results indicate that RanGAP1, p53, and pRB can be modified by SUMO-2/3. Interestingly, some slower migrated bands corresponding to polysumoylated p53 were also found, implying that p53 might be polysumoylated by SUMO-2/3.

The Sumoylation Site of p53 by SUMO-2/3 Has Been Identified to be Lys-386—To determine whether SUMO-2/3 also conjugates to p53 at Lys-386, HA-tagged wild-type p53 (p53WT) and HA-tagged mutant p53 (p53K386R) were transiently expressed in cells stably overexpressing SUMO-2/3GG (13–15). When His$_6$-tagged SUMO-2/3-conjugated proteins were isolated using Ni-NTA-agarose and analyzed by Western blot with anti-HA antibody, only p53 WT (but not p53K386R) was found to co-purify with His$_6$-tagged SUMO-2/3-conjugated proteins (Fig. 6A). These results indicate that SUMO-2/3 and SUMO-1 share the same conjugating site, Lys-386, in p53. However, no polysumoylated p53 was observed, likely because of the limited expression level of HA-tagged p53.

Oxidative Stress Stimulated p53 Sumoylation by SUMO-2/3 but Not SUMO-1—It has been reported that sumoylation by SUMO-2/3 (but not SUMO-1) responds to a number of stress conditions, e.g., heat shock and oxidative stress (8). As shown in Fig. 6B, when untransfected HEK293 cells were treated with 5 μM H$_2$O$_2$ for 20 min at 37 °C, the SUMO-2/3-modified p53 was elevated from an undetectable level to a clearly visible level in Western analysis monitored by anti-SUMO-2/3 antibody. However, no polysumoylated p53 was detected under these conditions. This observation is likely because of the presence of the relatively low abundance of modified p53. On the other hand, the level of SUMO-1-modified p53 exhibited no noticeable change following H$_2$O$_2$ treatment (Fig. 6B).

Increase of p53 Transcriptional Activation by Modification with SUMO-2/3—To assess the functional changes of p53 in cells resulting from SUMO-2/3 modification, reporter gene constructs containing binding sites for p53 at the promoter regions were used to analyze the transcriptional activity of p53. When stable cell lines were transfected with the pRGC-Luc p53 reporter plasmid, the luciferase activity in cells expressing SUMO-2GG was found to be elevated ∼4-fold over those expressing SUMO-2ΔGG. Similarly, luciferase activity in cells expressing SUMO-3GG was ∼2-fold over those expressing SUMO-3ΔGG. In contrast, both SUMO-2ΔGG- and SUMO-3ΔGG-transfected cell lines showed similarly low levels of luciferase activity (Fig. 7). Similar results were also observed when pG13-Luc was used as the p53 reporter plasmid or when MEF 3T3 Tet-Off cells were co-transfected with pRGC-Luc, p53WT, and the four SUMO-2/3 constructs (data not shown). In addition, p21, which is a direct target of p53, was induced in cells overexpressing SUMO-2/3 but not in those overexpressing the non-conjugatable SUMO-2/3ΔGG (Fig. 1). These findings suggest that overexpressing SUMO-2/3GG elevates the p53 expression levels of p53.
sumoylation level, which in turn stimulates p53 transcriptional activity.

DISCUSSION

Considering the distinct aspects of the elevation of protein modification by SUMO-2/3 in response to biological stresses (8), it is of interest to identify SUMO-2/3 target proteins and to investigate the consequences of these modifications. In this study, stable cell lines overexpressing SUMO-2/3GG were used to serve as models for evaluating the effects of the elevated level of SUMO-2/3 modification under stress conditions. As shown in the right panel of Fig. 1, the overall SUMO-2/3 conjugation patterns, except for the elevation of conjugation levels, are similar to endogenous SUMO-2/3 conjugation patterns in cells expressing non-conjugatable SUMO-2/3ΔGG. These results indicate that our stable cell lines could serve as suitable models mimicking the stress-induced elevation of SUMO-2/3 conjugation. We showed clearly that RanGAP1, the most abundant substrate of SUMO-1, is also sumoylated by SUMO-2/3 at their endogenous levels in HEK293 cells. (Fig. 2A) and that overexpression of SUMO-2 or SUMO-3 significantly elevated the level of SUMO-2/3-modified RanGAP1 (Fig. 1). This finding is different from a previous report that SUMO-2/3 conjugated poorly, if at all, to RanGAP1 (8). The discrepancy could result from the facts that COS-7 cells, which contain higher levels of free SUMO-2/3 relative to that in HEK293 cells, were used in the previous report and that a more sensitive anti-SUMO-2/3 antibody was used in this study (Fig. 2A).

The two-dimensional electrophoresis data for the three SUMO members showed that the global modification patterns of SUMO-2 and SUMO-3 were very similar, whereas they are much different from that of SUMO-1 (Fig. 2B), indicating that SUMO-1 and SUMO-2/3 may target different proteins or target common proteins with different extents of modification. Because there is only a three-amino-acid difference between the processed forms of SUMO-2 and SUMO-3 in humans, it is technically difficult to distinguish these two proteins. Thus, the functional differences of these two paralogues may derive from their differential activation processes. In addition, Northern blot analysis has revealed that SUMO-2 and SUMO-3 are tissue-specific (18, 19), whereas SUMO-1 is ubiquitously expressed in many tissues (20). During the physiological function study with stable HEK293 Tet-On cell lines overexpressing SUMO-2/3, we identified a...
number of SUMO-2/3 target proteins (21), and intriguingly, we observed that cells overexpressing SUMO-2/3GG (but not SUMO-2/3ΔGG) showed slow growth and cellular senescence phenotype. This observation is consistent with the report by Ayaydın and Dasso (22) revealing their failure to select stable HeLa cell lines expressing processed SUMO proteins, likely due to cellular intolerance to elevated levels of the processed forms of SUMO proteins. In view of the fact that HEK293 cells are adenovirus-transformed immortal cells and because it is therefore unusual for them to exhibit cellular senescence, we stably overexpressed SUMO-2/3GG in MEF 3T3 cells. As shown in Fig. 3, elevated expression of SUMO-2/3 indeed led to premature cellular senescence. In support of our original observation on cellular senescence induced by SUMO-2/3 overexpression, during the revision of the manuscript, Bischof et al. (23) reported that overexpression of the E3 SUMO ligase PIASy provoked cellular senescence. Together, these two observations strongly suggest that elevated sumoylation of certain target protein(s) causes premature cellular senescence. The p21/p53 and p16/pRB pathways are two major pathways known to be involved in senescence (10, 11). Our data show that the levels of p53 and pRB remain relatively constant, whereas the p21 level increases significantly in cells overexpressing SUMO-2/3GG but not in those overexpressing SUMO-2/3ΔGG (Fig. 1). These data imply that up-regulation of p21, a protein transcriptionally regulated by p53, may result from p53 modification by SUMO-2/3. Furthermore, knocking down either p53 or pRB significantly alleviated the premature senescent phenotypes caused by the elevation of SUMO-2/3 modification. Together, these results indicate that the senescence phenotype induced by overexpressing SUMO-2/3 is dependent on both the p53 and pRB pathways. The fact that both p53 and pRB could be modified by SUMO-2/3 (Fig. 5), suggests that p53 and pRB could be regulated via SUMO-2/3 modification. Consistent with these findings, Bischof et al. (23) also report that overexpression of PIASy in normal human fibroblasts recruits p53 and pRB tumor suppressor pathways to induce a senescence arrest. Interestingly, p53 (but not pRB and RanGAP1) was found to be polysumoylated, suggesting that polysumoylation by SUMO-2/3 is substrate-specific. The polysumoylated p53 was observed when the relatively high level of p53 in HEK293 cells was coupled with SUMO-2/3 overexpression (Fig. 5).

We showed that hydrogen peroxide treatment led to the increase of SUMO-2/3- but not SUMO-1-modified p53 (Fig. 6B), and overexpression of SUMO-2/3 significantly elevated p53 transcriptional activity (Fig. 7). These data suggest the potential involvement of SUMO-2/3 modification of p53 in response to oxidative stress. Concomitantly, there was up-regulation of p21 and an increase in the p53 transcription activity in cells overexpressing SUMO-2/3GG, whereas the level of p53 was not appreciably affected. Because one of the known senescence activation pathways is via the p53-induced elevation of p21 (24), it is not surprising that the sumoylation of p53 by SUMO-2/3 may contribute to premature senescence. The p53-mediated transcriptional increase in SUMO-2/3-overexpressed cells probably is not due to an increase in the stability of p53, because the p53 level was not significantly altered in cells overexpressing either processed or non-conjugatable SUMO-2/3. Covalent attachment of SUMO-2/3 to the C terminus of p53 (lysine 386) could conceivably alter p53-inherent transcription activity, subcellular localization, or target gene promoter binding.

Both our results and a previous report (8) showed that SUMO-1 exists mainly in its conjugated form with very low levels of free SUMO-1, whereas SUMO-2 and -3 are substantially in their free forms. Therefore, when cells are subjected to environmental stresses, such as heat shock or oxidative stress, SUMO-2/3 would be kinetically favorable for sumoylating their target proteins and regulating their functions. Moreover, as we reported previously (17), overexpression of SUMO-1GG did not cause apparent senescence phenotype. Together, the modification of p53 by SUMO-2/3 (but not SUMO-1) may serve as a signal in response to stresses, and the sumoylated p53 could promote the expression of its target genes leading to cellular responses such as senescence. However, the precise mechanism to up-regulating SUMO-2/3 modification during stress conditions remains to be elucidated. As SUMO-2/3 and SUMO-1 use the same E1 (activating) and E2 (conjugating) enzymes for conjugation, it is probable that either up-regulation of SUMO-2/3-specific E3 enzymes (ligases) or down-regulation of SUMO-2/3-specific proteases occurs in response to stresses. These studies also raise the interesting question of whether modification of p53 by different SUMO paralogues could regulate various sets of target genes. Further studies on p53 sumoylation may reveal new insights that could facilitate therapeutic developments.

Ledl et al. (16) report that pRB is modified by SUMO-1, and the viral oncoprotein E1A causes pRB desumoylation. The HEK293 cells are transformed with adenovirus protein E1A and E1B (25), and the expression of these viral proteins significantly inhibits the p53- and pRB-induced cellular senescence or apoptosis (26). The desumoylation of pRB is possibly a strategy used by viruses to suppress cellular senescence. Our results are consistent with the notion that overexpression of SUMO-2/3 may overcome the pRB desumoylation caused by E1A expression, and the elevated level of sumoylated pRB may subsequently participate in cellular senescence. The p53-induced apoptosis or senescence was also negatively regulated by the expression of E1A and E1B (27, 28). Whether sumoylation of p53 and pRB plays any role in overcoming the repression of apoptosis and senescence by E1A and E1B expression remains to be investigated.

In summary, our results demonstrate that the stable overexpression of SUMO-2/3 provides a reasonable model for mimicking the stress-induced elevation of SUMO-2/3-modified proteins. We revealed that both p53 and pRB, the most well-recognized tumor suppressor proteins, are sumoylated by SUMO-2/3. This sumoylation leads to an up-regulation of their activities on the transcription of target genes whose products, e.g. p21, may in part promote the observed cellular senescent phenotype. The distinct roles of SUMO-2/3 in the regulation of cellular senescence through the p53- and pRB-mediated pathway underscore the intricacy of the cellular sumoylation network.
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