

Fig. 1. (A) Schematic diagram of the siRNA expression cassette. The short hairpin form of siRNA is expressed under the control of a human U6 promoter. The five thymidines serve as a terminal signal. The sense and antisense siRNA strands are separated by a loop of 9 nt. Numbers indicate the positions of each target cDNA sequence. (B) Reduction of endogenous gene expression in Huh-7 cells transiently transfected by siRNA-expressing plasmids. Huh-7 cells were transfected with the indicated plasmids, and total RNAs were prepared at 48 h posttransfection. The mRNA level of La (upper left), PTB (upper right), eIF2B (lower left), and hVAP-33 (lower right) was analyzed by Northern blot with each specific probe. GAPDH served as a loading control.

PTB as cofactors for HCV IRES activity, which consequently suggests the utility of these host factors-targeting siRNAs in combating HCV infection. Simultaneously, the data presented here also suggest a functional relevance of hVAP-33 in both HCV and EMCV IRES-controlled translation initiation, which has not been reported previously. The mechanism underlying this observation and whether hVAP-33 is a universal internal initiation factor are to be further investigated.

Enhanced siRNA effect by adenoviral-mediated gene delivery

One important issue in utilizing siRNAs as therapeutic agents for human disease is how to deliver them to cells of

action. Among the gene delivery vehicles currently used in gene therapy approach, adenovirus is an attractive candidate for delivering siRNA against HCV, because it elicits long-lived transgene expression and allows targeting to the liver in vivo. Thus, we next cloned each siRNA expression cassette into recombinant adenovirus vector. Huh-NNRZ cells stably replicating the HCV subgenomic replicon were transduced with each siRNA-expressing adenovirus at the multiplicity of infection (MOI) of 80, 30, and 10, cells were harvested at day 3 postinfection. Target mRNAs and proteins were analyzed by Northern and Western blot analysis in parallel. Transduction of the siRNA-expressing adenoviruses dose-dependently reduced the expression of their respective target genes, and the mRNAs or proteins of endogenous genes were markedly diminished by infec-

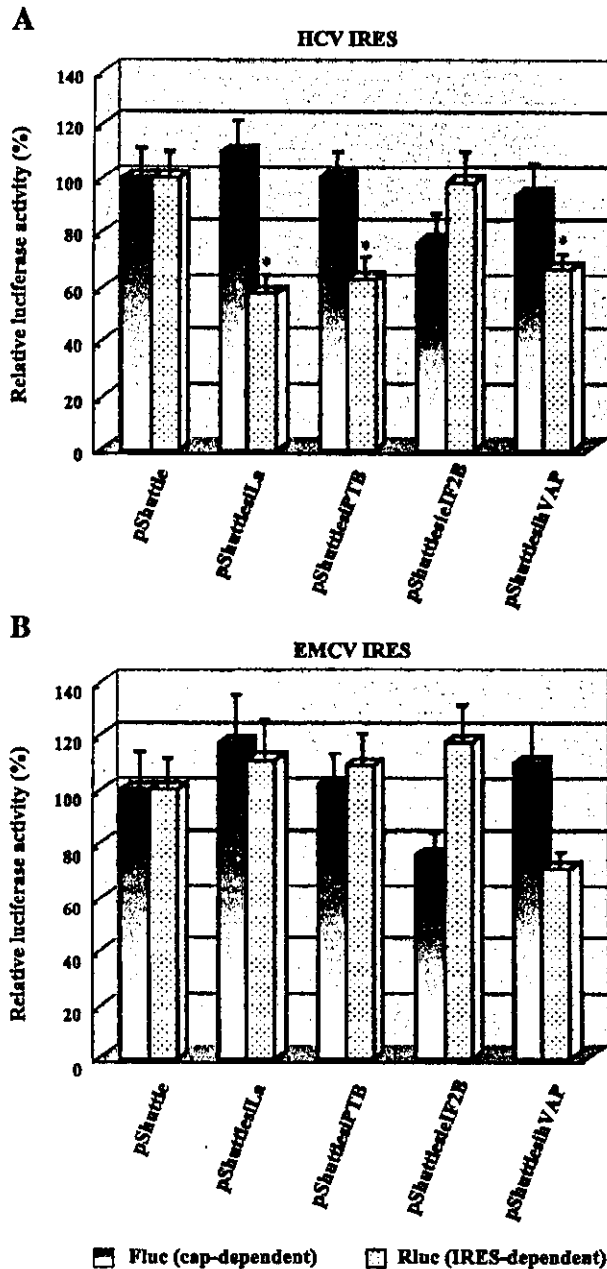


Fig. 2. Effect of endogenous genes silencing on HCV (A) or EMCV (B) IRES-dependent translation. HepT cells stably expressing T7 RNA polymerase were transfected with the reporter vector pNC371RL (A) or pEMCVRL (B), pGL3-Control vector, and the indicated siRNA-expressing plasmid. The cap-dependent firefly (Fluc) (shaded columns) and the IRES-dependent Renilla (Rluc) (dotted columns) luciferase activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of three independent triplicate transfections. * $P < 0.05$ compared with pShuttle.

tion of AdsiRNAs at the MOI ≥ 30 (Figs. 3A and B). While infection with the irrelevant AdsiLuc expressing siRNA against luciferase gene, even at the MOI of 80, had no effect on the expression of the target genes. Thus, adenovirally expressed siRNA elicits much more potent

RNAi activity when comparing with transient transfection of plasmids.

Effects of the endogenous gene silencing on HCV replication

To test the therapeutic potential of these siRNAs, we next investigate the effect of the endogenous gene silencing on HCV replication. Because HCV does not grow in cultured cells, we evaluated antiviral efficacy of the siRNAs in Huh-7 cells harboring an autonomously replicating HCV subgenome (Huh-NNRZ cells) (Kishine et al., 2002). Structurally, the subgenomic replicon in Huh-NNRZ cells contains the 5'-UTR, the first 36 nucleotides of the core region fused directly with the neomycin phosphotransferase gene, IRES element from EMCV, a nonstructural protein (NS) region from NS3 to NS5B and the 3'-UTR of HCV genome. The G418 resistance of Huh-NNRZ cells is conferred by persistent expression of neomycin phosphotransferase from replicating HCV RNAs. Thus, the ability of Huh-NNRZ cells to grow in G418-containing medium is an indirect measure of HCV replication. Huh-NNRZ cells were infected with AdsiLa, AdsiPTB, AdsiIF2B, AdsiHAP, or AdsiLuc at the MOI of 80, 30, and 10, and cultured in the absence or presence of G418 selection. After 10 days, the viable cells that were resistant to G418 because of the propagation of replicon were quantified with Cell Proliferation Reagent WST-1. In the absence of G418, viability of the cells transduced with each AdsiRNA was comparable to that of mock-infected cells (Fig. 4), except a moderate decrease in cells infected at the MOI of 80, probably reflecting some cellular toxicity or impaired survival due to higher degree of endogenous gene silencing. Following a 10-day exposure to G418, cells transduced with AdsiLa, AdsiPTB, and AdsiHAP showed a substantial and dose-dependent reduction in viable cell count, decreasing viable cell count by 99% and 97% at the MOI of 80 and 30, respectively. Significantly, even a modest reduction of La and PTB expression by infection of AdsiRNA at the MOI of 10 (Fig. 3) also resulted in substantial inhibition of HCV replication, leading to a 95% and 90% reduction in viable cell count, respectively. These results indicated that G418 resistance in most cells was lost due to the down-regulation of replicating HCV RNA by siRNA-mediated endogenous gene silencing. Consistent with the results from reporter assay, transduction with AdsiIF2B (Fig. 4) or irrelevant AdsiLuc (data not shown) had no significant effect on cell growth in the presence of G418. Because synthesis of the nonstructural proteins from the replicon is directed by EMCV IRES, silencing of cellular cofactor for EMCV IRES may also block the replication of HCV replicon. However, EMCV IRES-mediated translation was not affected by siLa or siPTB under the conditions in our study (Fig. 2B), so we conclude that significant loss of G418 resistance in replicon cell by depletion of La or PTB reflected a net interference effect of siLa and siPTB on HCV infection. In addition to

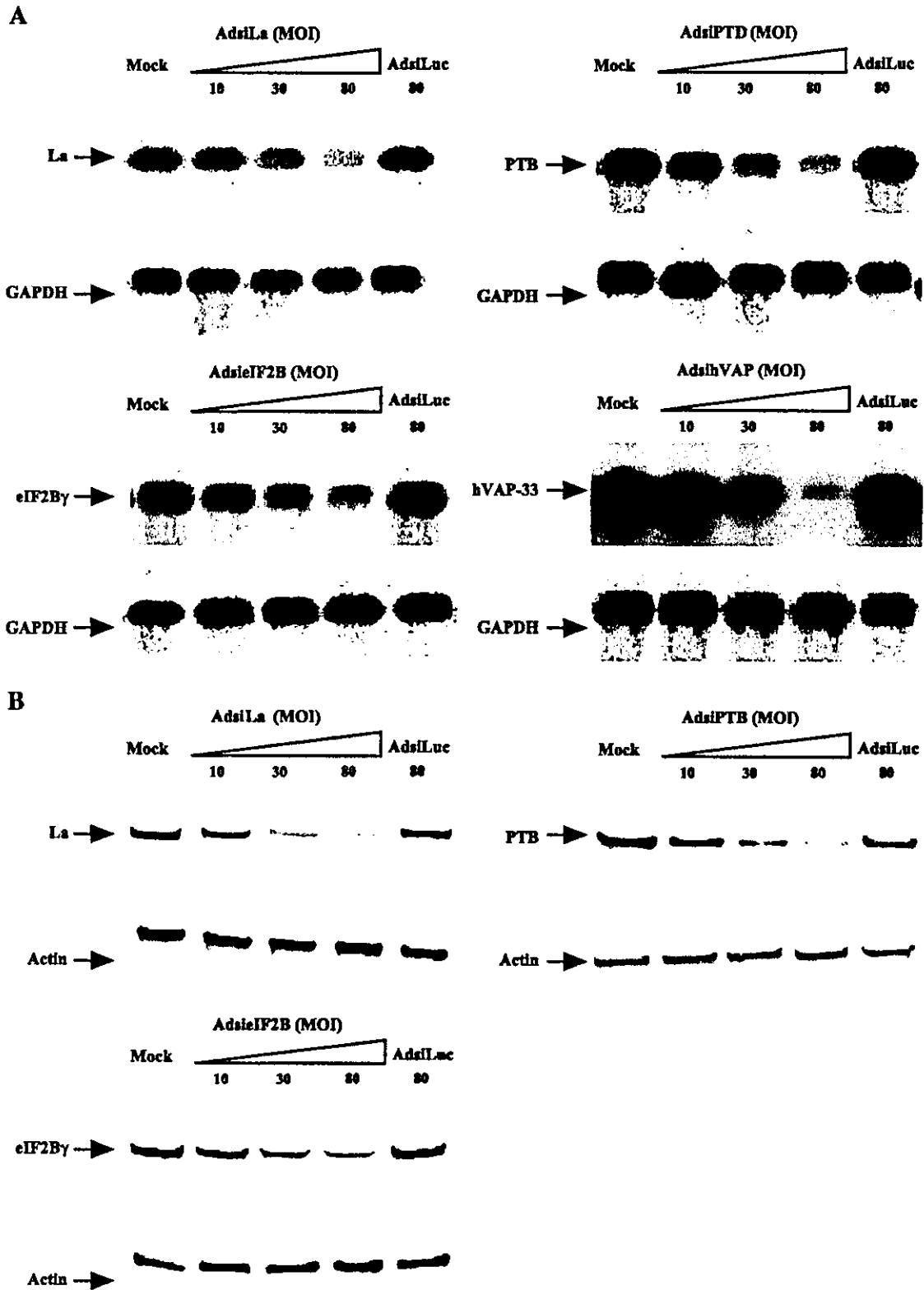


Fig. 3. Dose-dependent silencing of endogenous genes in Huh-NNRZ cells transduced with siRNA expression adenoviral vectors. Huh-NNRZ cells harboring HCV replicon were infected with each AdsiRNA at the MOI of 10, 30, and 80. The transduced cells were harvested at day 3 postinfection. The mRNA (A) and protein level (B) of the cellular factors were analyzed by Northern and Western blot. GAPDH and actin were served as controls for RNA and protein loading, respectively.

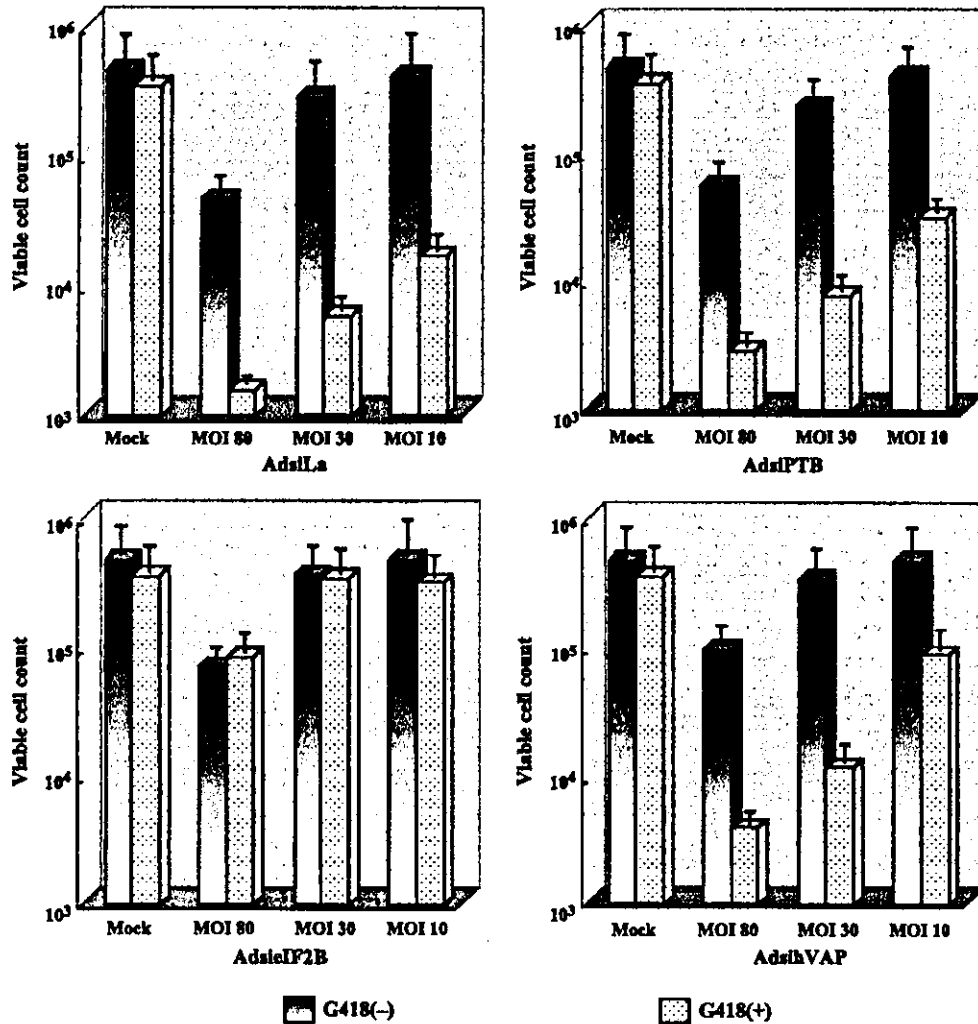


Fig. 4. Influence of the endogenous genes silencing on HCV replication. Huh-NNRZ cells coated on 12-well plate were infected with each AdsiRNA at the indicated MOI, and maintained either in the absence (shaded columns) or presence (dotted columns) of G418 selection. The viable cells were quantified after 10 days. Representative data are from three independent experiments.

translation, RNA replication may also be the target event for siRNA action, especially in the case of siPTB, because a specific interaction with 3'-UTR and a potential role in HCV minus-strand synthesis were reported for PTB. On the other hand, because both HCV and EMCV IRES-directed translation were inhibited by siRNA-mediated depletion of hVAP-33 (Fig. 2), the degree of antiviral effect of sihVAP may be overestimated here. Nonetheless, the results presented here demonstrate that siRNA-mediated silencing of the endogenous genes such as La, PTB, and hVAP-33 can efficiently inhibit HCV replication in cultured cells.

Discussion

Recently, a couple of laboratories have demonstrated RNAi-based inhibition of HIV, poliovirus, and HCV,

suggesting a potentially promising application of siRNA in antiviral therapy. However, it was shown that absolute conservation of the base-pairing in the siRNA target region is required for efficient cleavage, and if even a single nucleotide differs between an siRNA and its target, the effect is greatly diminished, or even eliminated completely. Therefore, in attacking HCV RNA directly, it is necessary to target viral RNA sequences that are conserved and normally invariant among different strains. Although the 5'-UTR and the 5'-end of core region are relatively conserved, considerable genetic diversity exists among various HCV isolates. The 5'-UTR region constitutes the IRES capable of initiating cap-independent translation of HCV. Highly ordered RNA structures and multiple sites participating in RNA-protein interaction within 5'-UTR have been documented to be critical for promoting internal initiation and/or its control, both of which might prevent recognition by siRNA. Thus, it may

be relatively difficult to choose optimal targets in HCV genome for therapeutic siRNA. Furthermore, because of the inaccuracy of RNA-dependent RNA polymerase, mutated progeny virus may rapidly emerge and escape from recognition by siRNA.

Here, we investigated whether siRNAs directed to cellular genes presumably essential for viral infection can inhibit HCV replication in cultured cells. Our results presented here showed that adenoviral-delivered siRNAs elicited potent RNAi effect on endogenous genes, and silencing of La, PTB, and hVAP-33 by AdsiRNA consequently blocked HCV replication in Huh-7 cells. Significantly, even a modest reduction of La and PTB expression by infection of AdsiRNA at the MOI of 10 also resulted in substantial inhibition of HCV replication, decreasing viable (infected) cells by 20- and 10-fold, respectively (Fig. 4). By demonstrating that the replication of the preexisting HCV replicon can be efficiently suppressed by cofactor-targeting siRNAs, it is conceivable that pretreatment of naive liver cells with these siRNAs could protect them from HCV infection. This would be particularly useful in preventing newly transplanted livers from reinfection with HCV.

Besides the attenuation of HCV IRES activity by siLa and siPTB, the effective anti-HCV effect may partially be attributed to the interference with HCV RNA replication. This is especially true in the case of siPTB, because PTB has been shown to bind to the 3'-end of the HCV RNA at two conserved stem-loop structures, which is critical for minus-strand synthesis (Tsuchihara et al., 1997; Ito and Lai, 1997). The data presented here showed a function relevance of hVAP-33 in HCV IRES-dependent translation, together with the previous study suggesting that hVAP-33 may participate in HCV replication by serving as a membrane receptor for replication complex (Tu et al., 1999), implicating that hVAP-33 may be a cellular factor playing a role in regulating the switch between translation and replication of HCV RNA. If this is the case, depletion of hVAP-33 by sihVAP may inhibit both translation and replication of HCV RNA, the processes which may be coupled, as that reported in poliovirus (Novak and Kirkegaard, 1994).

Different from the genomic structure of native HCV in which the viral polyprotein is translated under the control of its IRES, synthesis of the nonstructural protein in the replicon is initiated by EMCV IRES, whereas translation of the neomycin phosphotransferase is directed by HCV IRES. Theoretically, silencing of cellular factors, which are specific for HCV IRES, does not affect the expression level of nonstructural proteins, and consequently, replication of HCV replicon may not be altered. Accordingly, quantification of HCV RNA transcripts or protein expression would underestimate the authentic anti-HCV effect of the siRNAs against cofactors for HCV IRES. In view of this respect, we assessed anti-HCV efficacy of the siRNAs by measuring the loss of G418 resistance, which can be

taken as a comprehensive measure of HCV infection including both translation and RNA replication. Further experiments with other HCV-permissive cell culture or small animal models of chronic hepatitis C are required to validate the anti-HCV function of these siRNAs. Additionally, it is important to confirm that silencing of these endogenous genes by siRNA has no serious deleterious effects. La protein is an autoimmune antigen that is transiently associated with the 3' oligo (U) terminus of the RNA polymerase III transcripts and facilitates transcription termination and recycling of transcription complexes for reinitiation process (Maraia, 1996). One concern is whether La silencing may affect the transcription of tRNAs, 5SrRNA, and other transcripts synthesized by pol III. PTB is an RNA-binding protein recognizing pyrimidine-rich sequence, playing a role in regulating alternative splicing by selectively repressing 3' splice sites (Singh et al., 1995). Also unknown is whether knock-down of PTB may lead to perturbation of cellular RNA processing. hVAP-33 has a typical SNARE structure, potentially involved in diverse vesicle trafficking between membrane compartments, although its precise functions are unknown currently. One possibility is that partial depletion of hVAP-33 may affect the vesicle transport functions of cells. Further studies are required to determine whether this endogenous gene silencing is truly limited by its roles in cells. Nonetheless, the results presented here provide proof of principle for the idea that siRNA directed to cellular cofactor can serve as a potential therapeutic agent for HCV infection, which can be used either alone or in combination with siRNA directed to HCV RNA genome. It is theoretically possible that siRNA simultaneously targeting cellular and viral gene could help to prevent the escape of mutant variants and provide additive or synergistic effects.

In addition to the therapeutic significance, the siRNA-based approach allowed us to provide further evidence for the functional requirement of La and PTB in the HCV IRES-mediated translation, not only in the reporter system, but also in the context of HCV replicon. In contradiction to those reported by Kruger et al. (2000), our data do not support a role of eIF2B γ in regulating HCV IRES function. As shown in Fig. 4, the G418-resistant phenotype of replicon cells, which is conferred by HCV IRES-directed translation of neomycin phosphotransferase, was not affected by transduction with AdsiEIF2B, although a significant silencing of endogenous eIF2B γ was observed. These results substantially lessen, if could not rule out completely, the possibility that eIF2B γ is a cofactor for HCV IRES. The basis for the discordance in the data presented by Kruger et al. and those presented here is currently unknown. Unexpectedly, our data showed a biological relevance of hVAP-33 in both HCV and EMCV IRES-mediated translation. To our knowledge, involvement of hVAP-33 in IRES-dependent translation has not been reported previ-

ously. Whether hVAP-33 is a universal internal initiation factor that is indispensable in every case of internal initiation is to be further investigated.

In conclusion, the results presented here demonstrate that adenoviral-delivered siRNA specific to cellular genes such as La, PTB, and hVAP-33 significantly inhibit viral replication in cultured Huh-7 cells. These data also suggest that siRNA-based approach is a powerful tool for identification of novel HCV cellular cofactors yet to be described, thereby facilitating the development of new antiviral therapy for HCV infection.

Materials and methods

Plasmids

The U6 pol III promoter was amplified by PCR from the genomic DNA of the HeLa cells with a sense primer containing a *MfeI* site 5'-atgcaattgAAGGTCGGGCAG-GAAGA-3' and an antisense primer containing an *EcoRI* and a *BamHI* site 5'-agaattcggatccCGCGTCCTTTCCA-CAAGATA-3'. The PCR product was digested with *MfeI* and *EcoRI*, and cloned into the *MfeI* and *EcoRI* sites of pShuttle (Clontech) to create pShuttleU6. Target sequences for siRNAs were selected according to the Ambion web-based criteria and further analyzed by BLAST research to avoid a significant homology with other genes. For each endogenous gene (La, PTB, eIF2B γ , or hVAP-33), sense and antisense oligonucleotides of self-complementary hairpin sequences (including the termination signal of five thymidines), which contain cohesive ends for *BamHI* and *EcoRI* at the 5'- and 3'-ends, were synthesized and annealed by heating at 95°C for 10 min and slowly cooled down to room temperature. After a gel electrophoresis purification, these annealed oligonucleotides were inserted into the *BamHI* and *EcoRI* sites of pShuttleU6. The resultant vectors are termed pShuttlesiLa, pShuttlesiPTB, pShuttlesieIF2B, and pShuttlesihVAP, respectively. The sequences of these constructs were confirmed by nucleotide sequencing.

Adenovirus

Each siRNA-expressing cassette in pShuttlesiRNA, which is flanked by *I-CeuI* and *PI-SceI* sites, was digested with these two enzymes, and ligated to the E1- and E3-deleted Adeno-X Viral DNA (*I-CeuI* and *PI-SceI* digested) (Adeno-X Expression System, Clontech). The resultant adenoviral DNAs were digested with *PacI* and then transfected into low-passage 293 cells. Seven days following transfection, crude virus was prepared from the transfected cells by three cycles of freeze–thawing, and further amplified in 293 cells by several rounds of infection. The purified virus was aliquoted and stored at –80°C before use. The authenticity of all recombinant

adenoviral DNAs was verified before preparing high-titer viral stocks.

Cells

The cell lines Huh-7 and 293 were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 50 units/ml penicillin and streptomycin in a 5% CO₂ humidified atmosphere. The cell line HepT, which stably expresses T7 RNA polymerase (Zhang et al., 1999), was grown in DMEM containing 5 μ g/ml puromycin (Sigma). A Huh-7-derived cell line (Huh-NNRZ) stably replicating HCV subgenomic replicon (referred to as #50-1 cell clone in Kishine et al., 2002) was grown in DMEM containing 300 μ g/ml G418 (Geneticin, Invitrogen).

Northern and Western blot analysis

Total RNAs were isolated from culture cells with Trizol reagent (Invitrogen), separated by denaturing agarose gel electrophoresis, and analyzed by Northern blot using Dig-labeled probes. The probes correspond to nucleotides 72–581 of the cDNA for La, 88–656 for PTB, 87–598 for eIF2B γ , and 16–506 for hVAP-33. Western blot analysis was performed on total lysates from mock- or AdsiRNA-infected Huh-NNRZ cells. Protein was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel, transferred to Immobilon PVDF Transfer Membrane (Millipore). The blots were probed with monoclonal antibodies specific for La (clone La4B6, Progen Biotechnik GmbH), PTB (clone 1, Zymed Laboratories, Inc.), and eIF2B γ (sc-9980, Santa Cruz Biotechnology, Inc.). Proteins were visualized by using Immun-Blot Assay Kit (Bio-Rad).

Transfection

HepT cells were seeded onto 35-mm-diameter tissue culture dishes 24 h before transfection. Four micrograms of each pShuttlesiRNA, 2 μ g of the reporter vector, pNC371RL or pEMCVRL, and 0.2 μ g of pGL3-Control vector were cotransfected into HepT cells with TransFast Transfection Reagent (Promega). The cells were harvested after 48 h, and cell lysates were assayed for luciferase activity as described below.

Luciferase assay

Cell lysates were prepared from transfected cells, centrifuged briefly, and 20 μ l of the supernatants was used for luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a TD-20/20 Luminometer (Promega).

Quantification of cell viability

Cell viability was measured with Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's instructions.

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Transcriptional Activity of Peroxisome Proliferator-activated Receptor γ Is Modulated by SUMO-1 Modification*

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Covalent modification of many transcription factors with SUMO-1 is emerging as a key role of trans-activational regulation. Here, we demonstrate that peroxisome proliferator-activated receptor (PPAR) γ , which is a ligand-activated nuclear receptor, is modified by SUMO-1. Sumoylation of PPAR γ mainly occurs at a lysine residue within the activation function 1 domain. Furthermore, we show that the PIAS family proteins, PIAS1 and PIAS $\alpha\beta$, function as E3 ligases (ubiquitin-protein isopeptide ligase) for PPAR γ . PPAR γ interacts directly with PIAS $\alpha\beta$ in a ligand-independent manner. Analysis using a PPAR γ mutant with a disrupted sumoylation site shows that modification of PPAR γ by SUMO-1 represses its transcriptional activity. Interestingly, PIAS $\alpha\beta$ and Ubc9 enhance the transcriptional activity of PPAR γ independent of PPAR γ sumoylation. Furthermore, PPAR γ ligand-induced apoptosis in a human hepatoblastoma cell line, HepG2, is significantly enhanced by ectopic production of the sumoylation-mutant PPAR γ . These results suggest that the PPAR γ -dependent transactivation pathway seems to be modulated by SUMO-1 modification and may serve as a novel target for apoptosis-induction therapy in cancer cells.

PPAR γ ¹ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1) which regulates diverse biological functions including cell differentiation, growth inhibition, lipid metabolism, and apoptosis (2–5). Two isoforms of PPAR γ , PPAR γ 1 and PPAR γ 2, are generated by alternative promoter usage. PPAR γ 2, which contains an additional 30 amino acid residues at the amino terminus compared with PPAR γ 1, is predominantly expressed in adipose tissue,

whereas PPAR γ 1 is widely expressed (6).

The role of PPAR γ in adipogenesis has been extensively studied. Many adipocyte-specific genes, such as adipocytokines, contain PPAR γ -responsible elements in their promoter and/or upstream enhancer regions (7–10). PPAR γ plays a role as a central transcription factor in cellular differentiation and lipid accumulation during adipogenesis. Recent investigations demonstrate that treatment of a variety of human cancer cell lines with PPAR γ ligands leads to growth inhibition and apoptosis (2, 11–13). The use of PPAR γ ligands in the treatment of cancer is a potentially promising nontoxic and selective chemotherapeutic approach, and consequently, increased understanding of the mechanisms of PPAR γ in tumor suppression is needed.

Post-translational modifications regulate the function of many proteins. In the case of PPAR γ , transcriptional activity is reduced by mitogen-activated protein kinase-induced phosphorylation of serine residue 112 (14–16). Knock-in mice expressing PPAR γ with a Ser \rightarrow Ala mutation at this residue exhibit preserved insulin sensitivity in the setting of diet-induced obesity by changing fat cell size, generation of adiponectin, and increasing the amount of free fatty acid levels in serum (17).

Recently, a number of ubiquitin-like proteins (Ubl) have been identified that are covalently linked to lysine residues in target proteins (18, 19). One Ubl, SUMO-1, also known as PIC1, UBL1, sentrin, GMP1, and SMT3, is an 11-kDa protein that is structurally homologous to ubiquitin (20–22). SUMO-1 modification plays an important role in altering the function of modified proteins, including transcriptional activation, nuclear localization, and increased turnover (23–25). SUMO-1 is conjugated to proteins through a series of enzymatic steps (26). Initially, the ATP-dependent formation of a thioester bond between SUMO-1 and the E1 enzyme complex (SAE1-Uba2) is formed, and SUMO-1 is then transferred to the E2-conjugating enzyme Ubc9. Finally, SUMO-1 is conjugated from Ubc9 directly to a lysine residue of target proteins *in vitro*. The E3 ligase that conjugates SUMO-1 to target molecules *in vitro* and *in vivo* has only recently been identified (27–30). One group of such E3 ligases, protein inhibitor of activated STAT (PIAS) family proteins, homologous to the yeast Siz family protein, has a conserved RING finger domain that regulates transactivation of many transcription factors including STAT1 (31, 32), lymphoid enhancer factor-1 (33), and nuclear receptors (34, 35) by conjugating SUMO-1.

To understand the molecular mechanisms of PPAR γ transcriptional function through post-translational modifications, we explored the possible modification of PPAR γ by SUMO-1. In this paper we demonstrate that PPAR γ is a target for SUMO-1 modification, and PIAS proteins function as E3 ligases for SUMO-1 modification. The main sumoylation site of PPAR γ was mapped to a lysine residue at position 107, located in close proximity to the regulatory Ser-112. Sumoylation at this lysine residue reduced PPAR γ -dependent transcriptional activation

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¹ The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; GST, glutathione S-transferase; HA, hemagglutinin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; SUMO, small ubiquitin-like modifier; STAT, signal transducers and activators of transcription; PIAS, protein inhibitors of activated STAT; AF, activation function; HEK cells, human embryonic kidney cells.

significantly. However, reporter gene assays suggested that PIAS proteins enhanced the transcriptional activity of PPAR γ by a mechanism independent of PPAR γ sumoylation. We also observed that a PPAR γ sumoylation mutant displayed enhanced ligand-induced apoptosis in a human hepatoblastoma cell line, HepG2, which suggests a possible new target for cancer therapy.

MATERIALS AND METHODS

Cell Culture, Transfection, and Luciferase Reporter Assay—Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Transfection was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Luciferase activity was normalized to *Renilla* luciferase activity derived from co-transfected pRL-CMV-Luc (Promega). All reporter assays were performed in triplicate, and S.E. are denoted by bars in the figures.

Antibodies and Reagents—Rat anti-HA (3F10; Roche Applied Science), mouse and rabbit anti-FLAG (Sigma), and mouse anti-GFP (Clontech) antibodies were purchased commercially. Horseradish peroxidase-linked goat antibodies to rat IgG were from Jackson ImmunoResearch Laboratories. Horseradish peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences. Rosiglitazone was purchased from Alexis Biochemicals.

Plasmid Construction—Plasmids producing GST fusion proteins and pcDNA3 (Invitrogen)-based plasmids expressing epitope-tagged human SUMO-1, human UBC9, and mouse PIAS families have been described previously (36). A luciferase reporter plasmid, p4xPPRE-Luc, was constructed by inserting four copies of the PPAR response element (PPRE) (5'-TTGACCTTTGACCTTTGACCTTTGACCTTAGATC-3') into the luciferase reporter plasmid pGL2 (Promega). The mouse PPAR γ 2 gene containing the entire coding region was isolated by reverse transcription-PCR from 3T3-L1 cells and subcloned into epitope-tagged pcDNA3 to generate pcDNA3-FLAG-PPAR γ 2, -HA-PPAR γ 2. The cDNAs for mutant PPAR γ 2 with substitution of Lys-107 to Arg, PPAR γ 2(K/R1), and Lys-159 to Arg, PPAR γ 2(K/R2), were created using site-directed mutagenesis and subcloned into expression vectors to obtain pcDNA3-FLAG-PPAR γ 2(K/R1) and -PPAR γ 2(K/R2). The expression vector for PPAR γ 1 was obtained using PCR from PPAR γ 2 as a template. Expression plasmids of PPAR γ -GAL4 DNA-binding fusion proteins were generated by inserting these genes into pM (Clontech). pSG5-mRXR α , encoding mouse RXR, was kindly provided by P. Chambon of the Institut de Genetique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP/College de France, and S. Kato at the Institute of Molecular and Cellular Biosciences, The University of Tokyo.

GST Pull-down Analysis—GST and GST fusion proteins were expressed in the *Escherichia coli* strain BL21 (DE3) and affinity-purified with glutathione-Sepharose beads according to the manufacturer's instructions (Amersham Biosciences). PPAR γ 2 protein was metabolically labeled in the TNT-coupled reticulocyte lysate system (Promega) with T7 RNA polymerase and [³⁵S]methionine. GST pull-down analysis was carried out as described previously (37).

Immunoprecipitations—HEK-293T cells (1×10^6 per 6-cm-diameter dish) were transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. After incubation, cells were lysed in 1 ml of lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 5 mM EDTA, 10 mM *N*-ethylmaleimide, 200 μ M indole-3-acetic acid, and a complete protease inhibitor mixture tablet (Roche Applied Science) for sumoylation analysis or radioimmune precipitation assay buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM EDTA, and a complete protease inhibitor mixture tablet) for co-immunoprecipitation analysis. Cell debris was removed by centrifugation for 15 min. Lysates were first cleared with protein G beads for 30 min followed by incubation with antibodies for 1 h at 4 °C. Finally, the antibody complexes were captured with protein G beads for 1 h. Beads were washed four times with the same buffer, and immunoprecipitates were eluted and analyzed by Western blot.

Detection and Measurement of Apoptosis and Indirect Immunofluorescence Observation—HepG2 cells were grown to subconfluency on 8-well Lab-Tec Chamber (NUNC) in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were transfected with 500 ng of pcDNA3-FLAG-PPAR γ 1 or -PPAR γ 1(K/R1) expression vectors. Forty-eight hours after transfection, rosiglitazone was added to culture medium to a final concentration at 1 μ M for 24 h. Cells were fixed at room temperature with 3.7% formaldehyde for 3 min and then permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 5 min. After

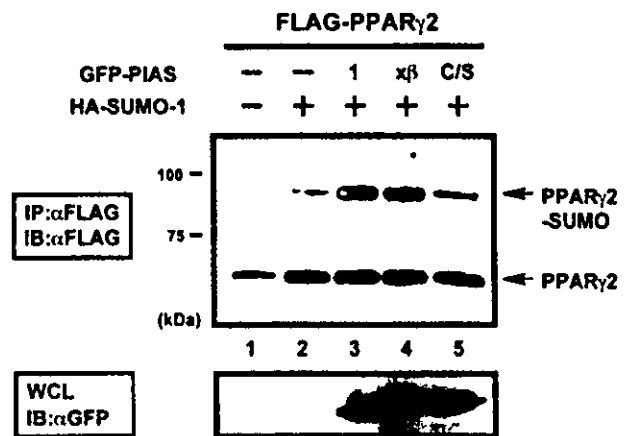
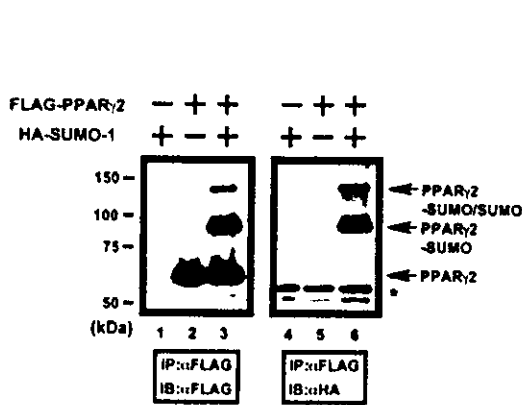
blocking with 3% bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline, the cells were incubated with anti-HA antibody for 1 h at 37 °C and stained with Alexa Fluor 568 anti-rat secondary antibody (Molecular Probes) for 1 h at room temperature. Apoptotic cells were detected by using the *in situ* cell death detection kit, Fluorescein, following the manufacturer's instructions (Roche Applied Science). The ratio of apoptotic cells was quantitated by analysis of DNA fragmentation using the cell death detection enzyme-linked immunosorbent assay according to the manufacturer's instructions (Roche Applied Science).

RESULTS

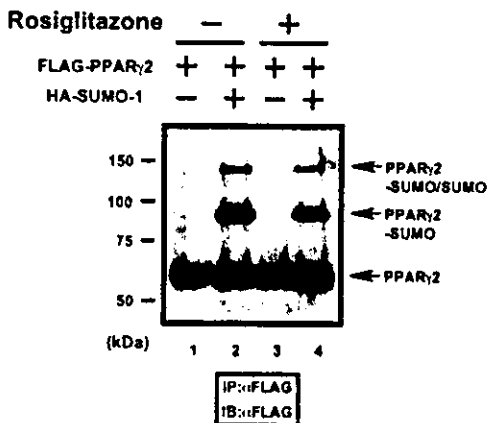
PPAR γ Is a Substrate for SUMO-1 Modification—SUMO-1 modification of certain transcription factors including nuclear hormone receptors is known to affect transcriptional activity, and consequently, we wished to address whether PPAR γ was a substrate for SUMO-1 modification. We first examined whether PPAR γ is modified by SUMO-1 in cells transiently expressing FLAG-PPAR γ 2 and HA-SUMO-1. Western blot analysis using anti-FLAG antibody revealed the presence of FLAG-tagged PPAR γ 2 in all cells transfected with the plasmid expressing FLAG-PPAR γ 2. When HA-SUMO-1 was co-expressed, additional slower migrating bands were detected by the FLAG antibody (Fig. 1A, left panel, lane 3). Moreover, to determine whether these slower migrating bands represent PPAR γ 2 conjugated to SUMO-1, the membrane was re-probed with anti-HA antibody, which detects proteins conjugated to HA-SUMO-1. The result showed that the slower migrating forms of PPAR γ 2, about 90 and 130 kDa, were indeed sumoylated (Fig. 1A, right panel, lane 6). These data suggest that PPAR γ is modified by SUMO-1 at least two sites. We next examined whether a specific PPAR γ ligand, rosiglitazone, affected PPAR γ sumoylation. As shown in Fig. 1B, SUMO-1-conjugated PPAR γ 2 was detected in cells co-producing SUMO-1. In lysates prepared from cells treated with rosiglitazone, the amount of SUMO-1-conjugated PPAR γ 2 was lower than in mock-treated cells (lane 2 and 4), suggesting that PPAR γ ligand negatively regulates SUMO-1 conjugation to PPAR γ . Two lysine residues, Lys-107 and Lys-347, in the AF1 and AF2 domains, respectively, of PPAR γ 2 conform to the proposed consensus motif ψ KX(D/E) (where ψ is a hydrophobic amino acid residue, X represents any residue, and D or E is an acidic residue) for SUMO-1-conjugating sites (18, 20) (Fig. 1C). To determine whether these lysine residues are targets for sumoylation, mutants with lysine to arginine substitutions, K107R (K/R1) and K347R (K/R2) as shown in Fig. 1C, were generated and analyzed for sumoylation. Two bands migrating slower than the original band were detected with almost the same intensity as cells producing both wild type PPAR γ 1, PPAR γ 2, and the mutant PPAR γ 2(K/R2) (Fig. 1D). In contrast, the slowest band disappeared in cells producing the mutant PPAR γ 2(K/R1), and moreover, the amount of the slower migrating band was also reduced in the cells expressing the mutant PPAR γ 2(K/R1) (Fig. 1D, lane 2). These results imply that Lys-107 is the major site for sumoylation, and this site may function as the master switch of sumoylation because mutation of this lysine residue greatly impaired sumoylation for PPAR γ . The fact that mutation of lysine residue at 347 did not affect the efficiency of sumoylation suggests the presence of lysine residues other than those not in the consensus motif for SUMO-1 modification in PPAR γ .

PIAS Family Proteins Act as E3 Ligases for PPAR γ Sumoylation—Recent studies indicated that members of the PIAS family enhanced sumoylation of many proteins including nuclear receptors (25, 34, 35). Therefore, we investigated whether PIAS family proteins function as E3 ligases for PPAR γ . We generated a mutant of PIAS β , PIAS β (C/S), in which the conserved cysteine residue at position 353 within the RING

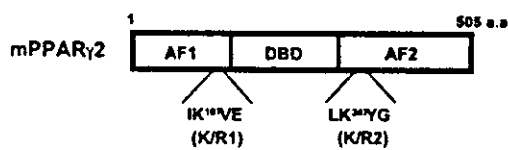
A



B



C



D

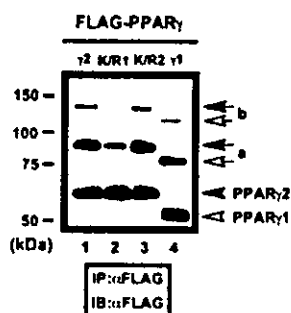


FIG. 1. PPAR γ is covalently modified by SUMO-1. A, HEK-293T cells were transfected with 2 μ g of each plasmid expressing FLAG-PPAR γ 2 together with (+) or without (-) 2 μ g of plasmid expressing HA-SUMO-1. Thirty-six hours after transfection, cell lysates were prepared and immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were subjected to 7.5% SDS-PAGE and analyzed by

FIG. 2. PIAS family proteins function as E3-ligases for PPAR γ . HEK-293T cells were cotransfected with 2 μ g of plasmid expressing FLAG-PPAR γ 2 together with (+) or without (-) 2 μ g of plasmid expressing HA-SUMO-1, GFP-PIAS1 (1), -PIAS α β (α β), or -PIAS α β (C/S) (C/S). Thirty-six hours after transfection, cell extracts were prepared and subjected to immunoprecipitation using anti-FLAG antibody (IP) followed by anti-FLAG immunoblot (IB). Levels of PIAS protein in whole cell lysates (WCL) are analyzed by immunoblot using anti-green fluorescent protein (GFP) antibody.

finger domain was changed to serine. This mutant was not able to interact with Ubc9 (data not shown) and completely lacked E3 ligase function (36). SUMO-1 conjugation to PPAR γ 2 was analyzed in cells producing either wild-type PIAS α β , PIAS α β (C/S), or PIAS1. Small amounts of SUMO-1-conjugated PPAR γ 2 were detected in cells expressing only HA-SUMO-1 ectopically (Fig. 2, lane 2). PPAR γ 2 sumoylation was enhanced by exogenous expression of PIAS1 and PIAS α β but not PIAS α β (C/S) (lanes 3–5). These findings indicate that PIAS family proteins function as E3 ligases for PPAR γ 2.

Next, to investigate the association of PPAR γ 2 with PIAS α β , we employed a GST pull-down analysis. Full-length PIAS α β expressed in bacteria as a GST fusion protein was coupled to glutathione *S*-Sepharose beads, and this complex was incubated with *in vitro* translated ³⁵S-labeled PPAR γ 2 in buffer with or without rosiglitazone, a specific ligand for PPAR γ . As shown Fig. 3A, PPAR γ 2 interacted both in the presence and absence of rosiglitazone with GST-PIAS α β but not with GST

immunoblot (IB) with anti-FLAG antibody (left panel). After ECL development, the membrane shown in the left panel was stripped and re-probed with anti-HA antibody (right panel). The asterisk indicates the immunoglobulin heavy chain. B, sumoylation of PPAR γ was repressed by ligand treatment. HEK-293T cells were transfected with (+) or without (-) 2 μ g of each plasmid expressing FLAG-PPAR γ 2 or HA-SUMO-1. Twenty-four hours after transfection cells were treated with (+) or without (-) 5 μ M rosiglitazone for 24 h. Cell extracts were prepared and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblot with anti-FLAG antibody. C, schematic representation of murine PPAR γ 2 (*mPPAR* γ 2). The AF1 domain, the DNA binding domain (DBD), and ligand-gated AF2 domain are shown. Two lysine residues, at positions 107 and 347, in the putative SUMO-1 acceptor sites are indicated below with the neighboring amino acid residues. *a.a.*, amino acids. D, PPAR γ was predominantly modified by SUMO-1 at Lys-107 within the AF1 domain. HEK-293T cells were cotransfected with 2 μ g of each plasmid expressing FLAG-wild-type PPAR γ 2 (γ 2), -mutant PPAR γ 2 with substitution of Lys-107 to Arg (K/R1), -mutant PPAR γ 2 with substitution of Lys-347 to Arg (K/R2), and FLAG-wild-type PPAR γ 1 (γ 1) together with plasmid expressing HA-SUMO-1. Thirty-six hours after transfection cell extracts were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblot with anti-FLAG antibody. Unmodified (arrowheads) and sumoylated (arrows with *a* and *b*) forms of PPAR γ 1 (white arrows and arrowhead) and PPAR γ 2 (black arrows and arrowhead) are indicated.

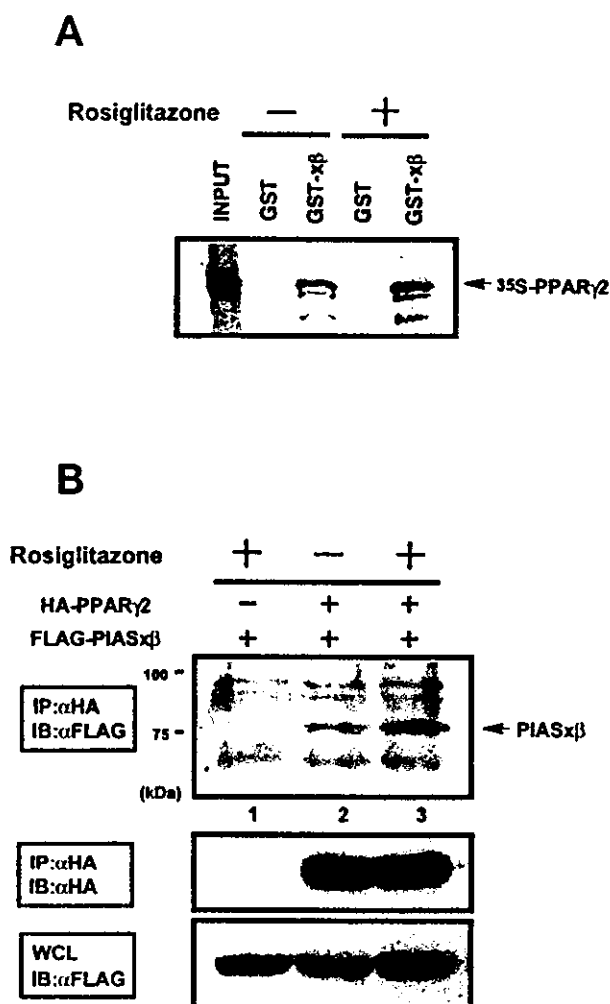


FIG. 3. Association of PIASx β with PPAR γ 2 *in vitro* and *in vivo*. **A**, GST and GST-PIASx β fusion proteins were immobilized on glutathione-Sepharose beads and incubated with 35 S-labeled PPAR γ 2 translated *in vitro* in pull-down buffer containing 5 μ M rosiglitazone as indicated. The pull-down complexes were analyzed by SDS-PAGE followed by an image analyzer. The *Input* lane represents 20% of total volume of whole cell lysates used for pull-down assay. **B**, HEK-293T cells were transfected with 2 μ g of plasmid expressing FLAG-PIASx β together with (+) or without (-) 2 μ g of plasmid expressing HA-PPAR γ 2. Twenty-four hours after transfection cells were treated with (+) or without (-) 5 μ M rosiglitazone for 12 h. Cell extracts were then prepared and subjected to immunoprecipitation using anti-HA antibody (*IP*). The immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblot (*IB*) with anti-FLAG antibody (*top panel*) or anti-HA antibody (*second panel*). The protein levels of FLAG-PIASx β in each cell lysate are indicated (*third panel*).

alone. To analyze the physical interaction of PPAR γ 2 with PIASx β in cells, a co-immunoprecipitation experiment was conducted using extracts from HEK-293T cells co-expressing HA-PPAR γ 2 and FLAG-PIASx β treated with or without rosiglitazone. Proteins precipitated with anti-HA antibody were resolved by SDS-PAGE and Western blot using anti-FLAG was conducted. A FLAG-reactive species was detectable in the complex precipitated with the anti-HA antibody. The PIASx β in the immunocomplex was increased in cells treated with rosiglitazone, indicating that the binding efficiency of PPAR γ 2 and PIASx β was significantly enhanced by treatment with this ligand (Fig. 3B, lanes 2 and 3).

SUMO-1 Conjugation to PPAR γ Represses the Transcriptional Activity of PPAR γ —To evaluate the effect of PPAR γ sumoylation on its transcriptional function, we analyzed the

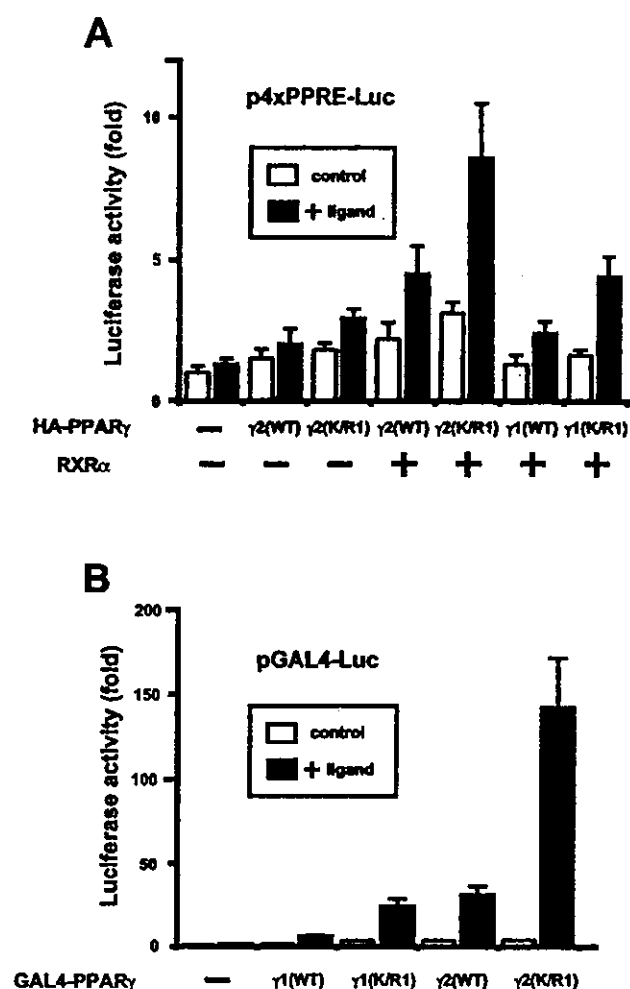


FIG. 4. Mutant PPAR γ has higher transcriptional activity than wild-type PPAR γ . **A**, NIH3T3 cells were cotransfected with 25 ng of p4xPPRE-Luc and 100 ng of each plasmid expressing HA-wild-type PPAR γ 2 (γ 2(WT)), -mutant PPAR γ 2 with substitution of Lys-107 to Arg (γ 2(K/R1)), -wild-type PPAR γ 1 (γ 1(WT)), -mutant PPAR γ 1 with substitution of Lys-77 to Arg (γ 1(K/R1)), mRXR α , or an empty plasmid (-). Twenty-four hours after transfection, cells were treated with or without 5 μ M rosiglitazone. Luciferase activities were then measured 18 h after treatment. *Open bars* denote no treatment, and *closed bars* indicate rosiglitazone treatment. The activity in control cells was arbitrarily given a value of 1, and the activities in the other cells were relative to the value of control cells. **B**, HEK-293T cells were cotransfected with 25 ng of GAL4-luciferase reporter plasmid (pGAL4-Luc) and 100 ng of plasmids expressing fusion proteins of the GAL4 DNA binding domain to PPAR γ 1 (γ 1(WT)), PPAR γ 1(K/R1) (γ 1(K/R1)), PPAR γ 2 (γ 2(WT)), PPAR γ 2(K/R1) (γ 2(K/R1)), or an empty plasmid (-). Twenty-four hours after transfection cells were treated with or without 5 μ M rosiglitazone. Luciferase activities were then measured 18 h after treatment. *Open bars* denote no treatment, and *closed bars* indicate rosiglitazone treatment. The activity in control cells was arbitrarily given a value of 1, and the activities in the other cells were relative to the value of control cells.

effects of PPAR γ sumoylation on expression of the p4xPPRE-Luc reporter gene in which the luciferase gene is driven by a PPAR-responsive promoter. NIH3T3 cells were transfected with various combinations of plasmids expressing wild-type PPAR γ 1, wild-type PPAR γ 2, PPAR γ 1(K/R1), PPAR γ 2(K/R2), and mRXR α , a component of a heterodimeric complex with PPAR γ , together with p4xPPRE-Luc. Cells were then treated with or without rosiglitazone. Additional production of mRXR α in cells enhanced reporter activity by PPAR γ . Reporter activity was enhanced by treatment with rosiglitazone, and this was

highest in cell lysates containing PPAR γ 2(K/R1) (Fig. 4A). The fact that transcriptional activity of PPAR γ 2 was higher than that of PPAR γ 1 was in good agreement with a previous report (38). Next, to analyze the direct effect of the SUMO-1 conjugation-dependent transcriptional activity of PPAR γ , we utilized PPAR γ fused with GAL4 to analyze gene expression from pGL2-Luc containing five GAL4 binding sites in the promoter region. GAL4-PPAR γ 1(K/R1) and -PPAR γ 2(K/R1) showed about 5-fold higher luciferase activities than the activity observed by GAL4-wild-type-PPAR γ 1 and GAL4-wild-type-PPAR γ 2, respectively, in a ligand-dependent manner (Fig. 4B). Taken together, these data suggest that sumoylation of PPAR γ represses the transcriptional activity of PPAR γ itself.

Both Ubc9 and PIAS α Enhance PPAR γ -dependent Transactivation—To further investigate the transcriptional role of sumoylated PPAR γ , we examined the effects of Ubc9, an essential factor for sumoylation, on PPAR γ -dependent transcription. GAL4-fused wild-type PPAR γ 2 and -PPAR γ 2(K/R1) were expressed in HEK-293T cells with increasing amounts of Ubc9 (Fig. 5A). Co-production of Ubc9 enhanced transcription by PPAR γ 2 and PPAR γ 2(K/R1) in a dose-dependent fashion. We next examined the effects of PIAS α and PIAS α (C/S) on the transcriptional activation of PPAR γ 2. Luciferase activities were significantly enhanced by the co-production of PIAS α . However, co-production of PIAS α (C/S) only slightly enhanced the activity (Fig. 5B). Similar results were also observed for PPAR γ 1 (data not shown). These data suggest that the SUMO-1 conjugation activity of Ubc9 and PIAS positively regulates PPAR γ -mediated transactivation. The observation that the transcriptional activity of PPAR γ 2(K/R1) was not only significantly enhanced by co-production of Ubc9 but also by PIAS α suggests that Ubc9 and PIAS proteins function as positive regulators for PPAR γ -dependent transcription possibly through SUMO-1 conjugation of a factor(s) other than PPAR γ involved in transcriptional regulation.

Ligand-induced Apoptosis by PPAR γ Is Enhanced in Cells Producing PPAR γ (K/R1)—Recent studies have demonstrated that specific ligands for PPAR γ inhibit cell growth and induce apoptosis in several human cancer cells (2, 11–13). PPAR γ activation seems to be important for inducing apoptosis in some cells. However, the molecular mechanisms of PPAR γ -dependent apoptosis, particularly the relationship between the transcriptional activity of PPAR γ and apoptosis, remain unclear. To investigate the effect of sumoylation on PPAR γ -dependent apoptosis, we compared the apoptotic potential of wild-type PPAR γ 1 to that of PPAR γ 1(K/R1) in HepG2 cells. Plasmids expressing FLAG-PPAR γ 1 or -PPAR γ 1(K/R1) were transfected into HepG2 cells, and 48 h after transfection cells were treated with 1 μ M rosiglitazone for 24 h. PPAR γ expression in cells and apoptotic cells were detected by immunostaining and terminal dUTP nick-end labeling assay, respectively. Approximately 5% of PPAR γ 1-transduced cells became terminal dUTP nick-end label-positive, which stained strongly by the anti-FLAG antibody. In contrast, ~40% of PPAR γ 1(K/R1)-transduced cells became terminal dUTP nick-end label-positive, and almost all cells expressed high levels of PPAR γ 1(K/R1) (Fig. 6A). The numbers of apoptotic cells producing PPAR γ 1 or PPAR γ 1(K/R1) were verified by measurement of the accumulation of fragmented nucleosomes. Ligand-induced apoptosis is significantly enhanced when PPAR γ 1(K/R1) was produced in cells (Fig. 6B). These results suggest that transcriptional activation of PPAR γ is involved in enhancing ligand-mediated apoptosis.

DISCUSSION

In this study we showed that sumoylation of PPAR γ significantly affected its transcriptional activity. PPAR γ was predominantly modified by SUMO-1 at Lys-107 within the AFI

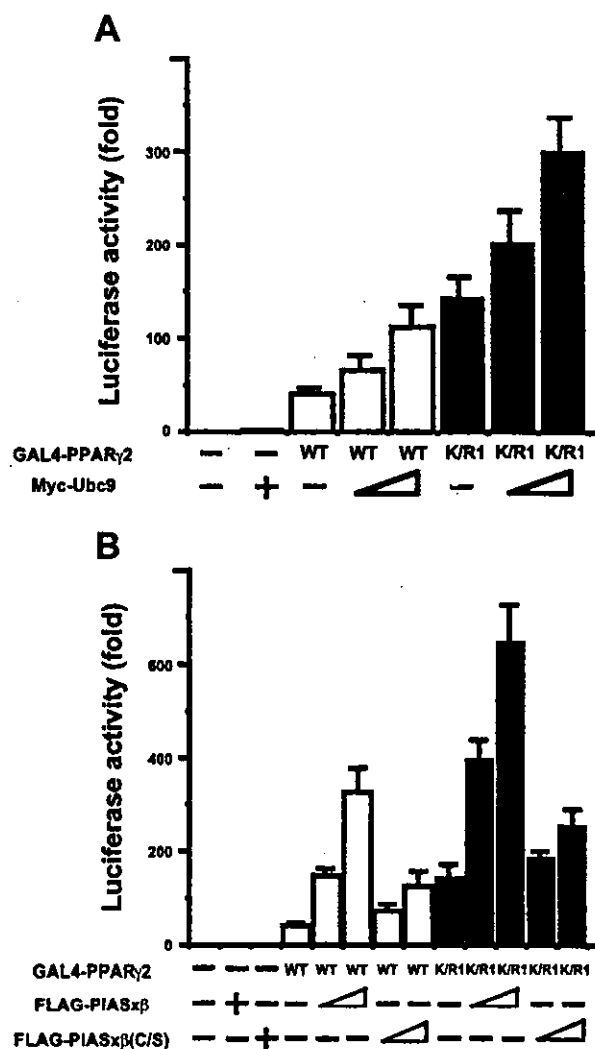


FIG. 5. PPAR γ -dependent transactivation is enhanced by Ubc9 and PIAS α independently of PPAR γ sumoylation. A and B, HEK-293T cells were cotransfected with 25 ng of pGAL4-Luc and 100 ng of plasmids expressing fusion proteins of the GAL4 DNA binding domain to PPAR γ 2 (WT), PPAR γ 2(K/R1) (K/R1), or an empty plasmid (-) together with 50 or 100 ng of plasmids expressing Myc-Ubc9, FLAG-PIAS α , or -PIAS α (C/S), respectively. Twenty-four hours after transfection cells were treated with 5 μ M rosiglitazone. Luciferase activities were then measured 18 h after treatment. The activity of the reporter plasmid alone in control cells was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this assay.

domain. Our result suggest that there is a lysine residue(s) in addition to Lys-107 targeted for sumoylation that is likely to lie in a non-consensus SUMO-1 conjugation motif, because mutational analysis of the lysine residues lying in other consensus SUMO-1 conjugation motifs in this protein did not affect SUMO-1 conjugation (Fig. 1D). Because mutation of Lys-107 reduced SUMO-1 conjugation of PPAR γ severely, Lys-107 is the primary site for modification. Similar observations of the presence of hierarchic lysine residues for SUMO-1 conjugation were reported in other proteins such as promyelocytic leukaemia protein (39), androgen receptor (40), aryl hydrocarbon receptor (41), and DNA topoisomerase I (42). PIAS1 and PIAS α acted as E3 ligase factors for SUMO-1 conjugation to PPAR γ (Fig. 2). We also showed that PIAS α associated with PPAR γ 2 *in vitro* and *in vivo* in a ligand-independent manner, but the

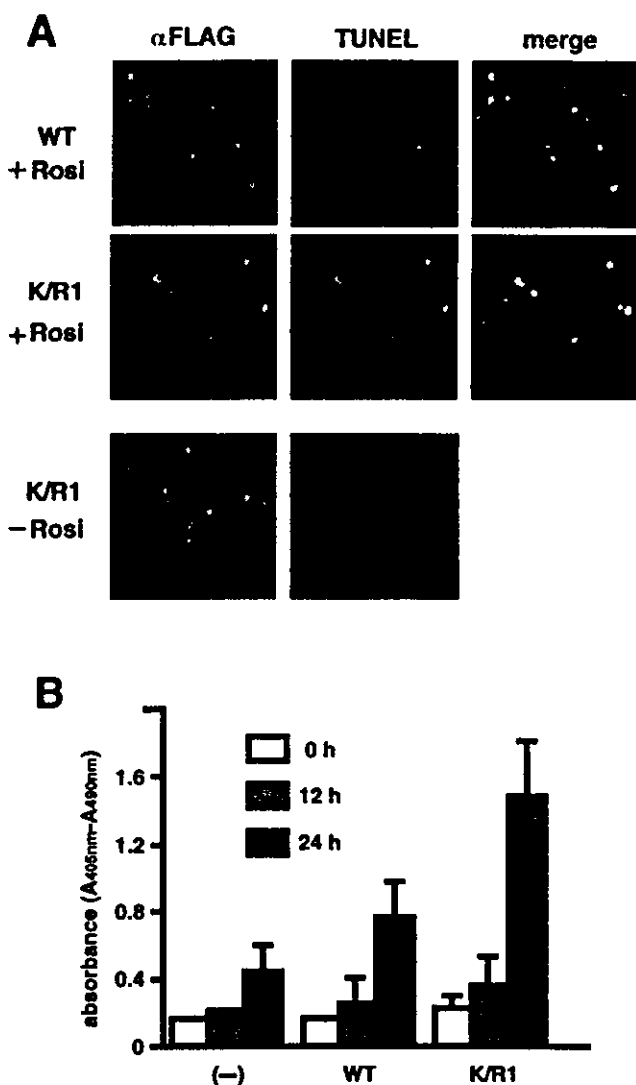


FIG. 6. Comparison of ligand-induced apoptosis between wild-type (WT) and sumoylation mutant PPAR γ 1 in HepG2 cells. *A*, HepG2 cells were transfected with plasmids expressing FLAG-PPAR γ 1 (WT), FLAG-PPAR γ 1(K/R1) (K/R1). Forty-eight hours after transfection cells were treated with (+Rosi) or without (-Rosi) 1 μ M of rosiglitazone for 24 h. Terminal dUTP nick-end labeling (TUNEL) assay and immunostaining were performed as described under "Materials and Methods." *B*, HepG2 cells were transfected with plasmids expressing FLAG-PPAR γ 1 (WT), FLAG-PPAR γ 1(K/R1) (K/R1), or an empty vector (-). Forty-eight hours after transfection cells were treated with 1 μ M rosiglitazone. Cells were then collected for 0, 12, and 24 h after treatment, and the ratio of apoptotic cells was quantitated by analysis of accumulation of fragmented nucleosomes. Open bars denote 0 h, shaded bars denote 12 h, and closed bars denote 24 h after treatment.

association was enhanced by the presence of the ligand *in vivo* (Fig. 3). Interestingly, ligand treatment led to a reduction in the amount of SUMO-1 conjugated to PPAR γ 2 (Fig. 1B). Because of conformational alteration of nuclear receptors, association of co-activator complexes with nuclear receptor seems to be regulated by specific ligands. Thus, it is likely that PPAR γ sumoylation is suppressed by the association of the co-activator complex with the ligated PPAR γ , in which the sumoylation sites of PPAR γ may be masked, and/or the E3 ligase activity of PIAS proteins may be blocked.

Using a reporter gene assay, we demonstrated that the transcriptional activity of PPAR γ was negatively regulated by sumoylation. It has been reported that phosphorylation of Ser-112,

adjacent to the sumoylation site, as revealed by this work, on PPAR γ by mitogen-activated protein kinase significantly inhibited both ligand-independent and ligand-dependent transcriptional activation by PPAR γ (15). Mutation analysis of the phosphorylation site revealed that this phosphorylation-mediated transcriptional repression was not due to a reduced capacity to make PPAR γ -RXR α complexes or the impairment of recognition of its DNA binding site (14). An AF-1 domain of PPAR γ may be negatively regulated by phosphorylation and sumoylation. Alternatively, SUMO-1-conjugated PPAR γ may recruit the transcriptional repressor complex by providing a novel interaction site. Recently it has been shown that sumoylation of the ETS domain transcription factor, Elk-1, results in the recruitment of histone deacetylase activity to promoters (43). Similarly, SUMO-1-conjugated PPAR γ may recruit additional cellular factors that repress PPAR γ -dependent transcription.

Our data clearly showed that Ubc9 enhanced the transcriptional activities of both PPAR γ and PPAR γ (K/R1), possibly by a mechanism independent of SUMO-1 conjugation to PPAR γ . PIAS α also enhanced PPAR γ activity through a RING finger domain-dependent mechanism. Thus, it seems that ectopically produced PIAS α regulates PPAR γ -mediated transactivation through not only sumoylation of PPAR γ itself but also in the conjugation of SUMO-1 to another cellular factor(s) involved in transcriptional regulation. In agreement with our observations, key molecules in the SUMO-1 conjugation system, including SUMO-1, Ubc9, and PIAS, have been shown to modulate the transcriptional activities of p53 (44), androgen receptor (40), aryl hydrocarbon receptor (41), and lymphoid enhancer factor-1 (33) even when these target molecules lacked a major sumoylation site(s) by mutation. Moreover, it has been shown that Ubc9 modulates the transcriptional activity of ETS-1- and TEL-independent of its E2 enzymatic activity (45, 46). In view of these reports, a mechanism(s) other than the direct SUMO-1 conjugation to PPAR γ by Ubc9 and PIAS α seem to be important for the regulation of transactivation of PPAR γ . Further studies to clarify the molecular basis of the transcriptional activation of PPAR γ -dependent transcription by Ubc9 and PIAS should provide significant insight.

A role for PPAR γ in adipogenesis is well characterized. In addition, a novel function of PPAR γ in tumor pathogenesis has been reported recently, which includes PPAR γ ligand-dependent growth inhibition and/or apoptosis in a variety of human cancer cells (2, 11-13). Several studies have demonstrated that induction of apoptosis was accompanied by the up-regulation of several pro-apoptotic genes, *Bax* and caspase-3 and -9, and down-regulation of the anti-apoptotic gene *Bcl-2* (47, 48), suggesting that transactivation of PPAR γ is likely to regulate expression of apoptosis modulators at the transcriptional level and contribute as an important modulator of tumor suppression. In this study we demonstrated that the trans-activation function of PPAR γ was up-regulated by mutation of Lys-107, the major target for sumoylation in PPAR γ . Because HepG2 cells expressing this mutant form of PPAR γ displayed enhanced PPAR γ ligand-dependent apoptosis, the increased transactivation function of PPAR γ seems to play an important role in inducing apoptosis. Sumoylation regulates the transacting function of PPAR γ , which could play a role in the regulation of apoptosis. We suggest here that the sumoylation of PPAR γ may be a good target for a novel therapeutic agent in cancer cells.

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Association of Ubc9, an E2 ligase for SUMO conjugation, with p53 is regulated by phosphorylation of p53

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Abstract Small ubiquitin-like modifier-1 (SUMO-1) conjugation to the tumor suppressor protein p53 seems to be regulated by murine double minute 2 homologue (Mdm2). It is thought that the physical association of Mdm2 with p53 is important for the enhancement of SUMO-1 conjugation to p53. However, mutant p53 that does not associate with Mdm2 is still sumoylated, albeit at a reduced level, suggesting that sumoylation of p53 is independent of the presence of Mdm2 and there is a direct association of ubiquitin-conjugating enzyme 9 (Ubc9), an E2 ligase for sumoylation, with p53. Here, we report evidence of the direct interaction of Ubc9 with p53. Furthermore, we observed that the interaction of Ubc9 with p53 was regulated by phosphorylation of p53. In particular, in cells treated with adriamycin that is a DNA damaging agent and that enhances phosphorylation of p53 at Ser-20, SUMO conjugation of p53 was severely impaired possibly by reduced affinity of Ubc9 to p53.

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Keywords: Small ubiquitin-like modifier conjugation; p53; Murine double minute 2 homolog; Ubiquitin-conjugating enzyme 9; Phosphorylation

1. Introduction

The tumor suppressor p53 plays a significant role in the cellular response to genome damage. The importance of p53 in maintaining genome integrity is emphasized by the high frequency of p53 mutations observed in many human tumors [1] and the high rate of tumor development seen in p53 knockout mice [2].

Although the level of p53 in cells is low under normal conditions, exposure to stress signals such as DNA damage and heat shock results in an increase in p53 levels because of its prolonged half-life [3]. The accumulation of p53 is responsible for increased transcription of p53 responsive genes, including proteins involved in cell-cycle regulation (e.g., p21^{waf1}) and apoptosis (e.g., Bax) [4–8]. Murine double minute 2 homo-

logue (Mdm2), also a p53 responsive gene, is a critical negative regulator of p53 [9,10].

The ubiquitin-proteasome system is responsible for the degradation and rapid turnover of p53 [11], and this is mediated through the association of p53 with Mdm2 [12–14]. The interaction of Mdm2 and p53 plays a critical role in suppressing the transcriptional activity of p53 [15–17], as well as the ubiquitination and degradation of p53 [18].

The role of ubiquitination in protein degradation is well documented and it has recently become clear that a small ubiquitin-like protein, variously known as small ubiquitin-like modifier 1 (SUMO-1)/sentrin/GMP1/UBL1/PIC1, can be covalently linked to a variety of cellular proteins [19–22]. p53 has been found to be covalently modified by SUMO-1 in vitro and in vivo at lysine 386 [23,24]. This sumoylation is enhanced by the association of E3 ligases, PIAS family proteins [25,26]. However, SUMO-1 and ubiquitin modification do not occur on the same lysine residue in p53 although they do in the case of IκBα [27]. Also, SUMO-1 conjugation to p53 does not seem to alter its transcriptional activity. Some reports suggest increased p53 activity upon sumoylation, but this remains controversial.

Enhanced p53 sumoylation occurs through direct interaction with Mdm2 in cells [28] and a p53 mutant that does not interact with Mdm2 is poorly sumoylated. An Mdm2 mutant with a constitutively activated cryptic nucleolus localization signal targets p53 to the nucleolus and promotes p53 sumoylation. These data suggest that enhanced sumoylation of p53 by Mdm2 is mediated by targeting p53 to the nucleolus through the formation of the Mdm2/p53 complex. Additionally, the phosphorylation state of p53 affects its ability to interact with Mdm2. It is known that DNA-dependent protein kinase, which targets serine residues 15 and 37, reduces the affinity between p53 and Mdm2, and as a consequence, phosphorylated p53 is thought to be a poor substrate for ubiquitination and sumoylation under these conditions.

To clarify the phosphorylation sites on p53 that affects sumoylation, we focused on the Ser-20, a target of the checkpoint kinase 2 (Chk2) activated by DNA damage [29]. In addition, we analyzed the sumoylation of p53 mutants with serine to glutamic acid substitutions at residues 46 or 392, a change thought to mimic the phosphorylated state of p53.

2. Materials and methods

2.1. Cell line

HEK-293T cells (adenovirus-transformed human embryo kidney cell line containing endogenous p53) were maintained in DMEM (Nissui)

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Abbreviations: Mdm2, murine double minute 2 homologue; SUMO, small ubiquitin-like modifier; Ubc9, ubiquitin-conjugating enzyme 9; GST, glutathione S-transferase; HA-tag, hemagglutinin epitope tag

supplemented with 10% fetal calf serum (MBL) and 200 $\mu\text{g/ml}$ of kanamycin (Meiji) at 37 °C in 5% CO₂ atmosphere.

2.2. Plasmid construction

A pcDNA3 (Invitrogen) based plasmid expressing FLAG-tagged human p53 (wild type) was provided by Dr. Y. Ariumi. The p53 mutants were produced individually using the site-directed mutagenesis system, Mutan Super Express Km (Takara), together with the generated pkF18K-p53 as a template. Mutagenetic oligonucleotides (Invitrogen) used in LA-PCR were: p53S20A, 5'-pGGAAACATTTGCAGACCTATG-3'; p53S20E, 5'-pCAGGAAACATTTGAAGACCTATG-3'; p53S46E, 5'-pGATTTGATGCTG-GAGCCGGACG-3' and p53S392E, 5'-pCAGAAGGGCTGACGAAGACTGACATTCTCCAC-3'. All plasmids were sequenced to confirm successful mutagenesis (ABI prism). FLAG-tagged p53 mutants were subcloned into pcDNA3. Plasmids encoding Myc-tagged ubiquitin-conjugating enzyme 9 (Ubc9) and hemagglutinin epitope tag (HA)-tagged SUMO-1 were generated as described previously [30].

2.3. Immunoprecipitations

HEK-293T cells (1×10^5 per 6 cm-diameter dish) were transfected using FuGENE6 (Roche) according to the manufacturer's instructions. To detect the sumoylated forms of p53, cells were lysed in 1 ml of RIPA buffer [25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol (DTT), 5 mM ethylenediamine tetra-acetic acid (EDTA), 10 mM *N*-ethylmaleimide, 200 mM indole-3-acetic acid, and a complete protease inhibitor cocktail tablet (Roche)] for 30 min on ice. Cell debris was removed by centrifugation for 15 min. Lysates were pre-cleared with protein G beads for 30 min, followed by incubation with antibodies for 1 h at 4 °C. Finally, the antibody complexes were captured with protein G beads for 1 h. Beads-bound proteins were washed four times with RIPA buffer, and immunoprecipitates were eluted and analyzed by immunoblot (IB). For co-immunoprecipitations, cells were transfected with 2 μg pcDNA3-FLAG-p53 expression plasmids with or without 2 μg pcDNA3-Myc-Ubc9 expression plasmid. After 36 h of culture, cells were lysed in 1 ml of immunoprecipitated (IP) buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM EDTA, and a complete protease inhibitor cocktail tablet]. Lysates were analyzed by immunoprecipitation followed by IB assay.

2.4. Adriamycin treatment

HEK-293T cells were transfected with the expression plasmid encoding FLAG-tagged p53WT. After 24 h of culture, cells were treated with 0.64 μM of adriamycin for another 24 h, and cell lysates were prepared for immunoprecipitation and IB assay.

2.5. Materials

Rat anti-HA (3F10, Roche), mouse anti-Myc (9E10, Santa Cruz), mouse and rabbit anti-FLAG (Sigma) antibodies were purchased. Phospho-Ser20-p53 antibody was generously provided by Dr. Y. Taya. Horseradish peroxidase (HRP)-linked goat antibodies to rat IgG were acquired from Jackson ImmunoResearch Lab. HRP-linked goat antibodies to mouse or rabbit IgG were purchased from Amersham Biosciences. Adriamycin was purchased from Sigma.

3. Results and discussion

To examine the sumoylation of FLAG-tagged p53, plasmids encoding wild-type or mutant p53 and HA-tagged SUMO-1 were transiently co-expressed in cells. Expression of FLAG-tagged p53 was confirmed by immunoprecipitation followed by immunoblotting. Two bands, one with the expected size of wild-type FLAG-p53 and another more slowly migrating band, were detected (Fig. 1). The apparent molecular weight of the upper band was higher in cells exogenously expressing SUMO-1. Because the upper band was detected by anti-HA, this band was the sumoylated form of p53 (Fig. 1, middle panel). Cells expressing p53S46E and p53S392E were similar to wild-type p53, but in the cells producing p53S20E, the upper band was very weak. This was further confirmed by the observation that the upper band in cells expressing p53S20E, but not p53S20A, was detected as a weak signal (Fig. 1, lower panel). These data suggested that phosphorylation of p53 at Ser-20 severely impaired SUMO-1 conjugation. Previously, it was shown that sumoylation of p53 was affected by its interaction with Mdm2 in cells [28]. A p53 mutant that poorly binds Mdm2 undergoes deficient sumoylation. These data suggest that Mdm2 plays an important role in the enhancement of p53 sumoylation. Since a p53 mutant that does not interact with Mdm2 is still sumoylated *in vitro*, Mdm2 does not seem to be an essential component for the sumoylation of p53 but rather enhances sumoylation. Since Ubc9, the E2 ligase for the SUMO-conjugation reaction, was shown to associate with a target molecule for sumoylation, the association of Ubc9 with

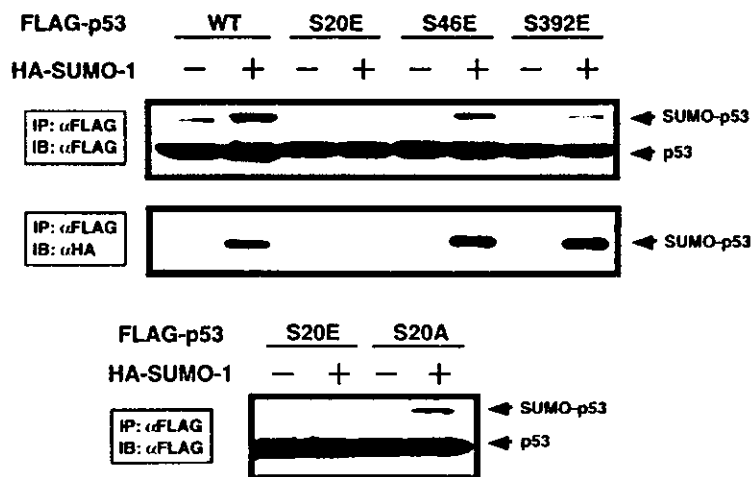


Fig. 1. Phosphorylation mimicked form of p53 at Ser-20 suppresses SUMO-1 conjugation. HEK-293T cells were transfected with 2 μg of plasmid expressing FLAG-tagged wild-type p53 (WT), or the p53 mutants S20E, S20A, S46E, or S392E with (+) or without (-) plasmids expressing HA-SUMO-1. Thirty six hours after transfection, cell lysates were prepared and IP with anti-FLAG mouse monoclonal antibody. The immunoprecipitates were subjected to 7.5% SDS-PAGE followed by analysis by IB using the anti-FLAG rabbit polyclonal antiserum (top panel). After stripping the filter shown in the top panel, the same filter was re-probed with the anti-HA antibody (middle panel).

p53 was examined. p53 was synthesized by in vitro translation in the presence of ³⁵S-methionine. The interaction of Ubc9 with ³⁵S-labeled wild-type p53, p53S20E and p53S20A was analyzed by GST pull-down analysis (Fig. 2A). Ubc9 bound all the p53 products tested with slightly varying affinity. The affinity of Ubc9 towards p53S20E was reduced by half (Fig. 2B). Because there is no Mdm2 in this assay system, there appears to be a direct interaction between Ubc9. The interaction of Ubc9 and p53 was also observed in vivo (Fig. 2C). Cells were co-transfected with Myc-tagged Ubc9 together with FLAG-tagged p53, p53S20A or p53S20E. Cells lysates prepared from these cells were then analyzed by immunoprecipitation followed by IB. Comparing to the amount of wild-type p53 and p53S20A in the complexes co-precipitated with Ubc9, that of p53S20E was significantly reduced. However, this result may not support the possible direct interaction of p53 with Ubc9, since Ubc9 is known to interact with Mdm2.

The Ser-20 of p53 can be directly phosphorylated by Chk2 in response to DNA damage [29]. To address whether sumoylation of p53 is suppressed by phosphorylation of p53 at Ser-20, we analyzed the sumoylation of p53 after adriamycin treatment. In order to determine whether adriamycin treatment led to phosphorylation of Ser-20 of p53, HEK-293T cells were transfected with a plasmid expressing FLAG-tagged wild-type p53 and treated with 0.64 μM of adriamycin at 24 h post-transfection. Adriamycin treatment was performed for 24 h and the whole cell lysates were prepared for analysis by IB assay with phospho-Ser20 p53 antibody (Fig. 3, upper panel). Ser-20 phosphorylation following adriamycin treatment was observed. We next analyzed sumoylation of p53 in cells treated with adriamycin. Sumoylation of p53 was significantly decreased after adriamycin treatment (Fig. 3, lower panel),

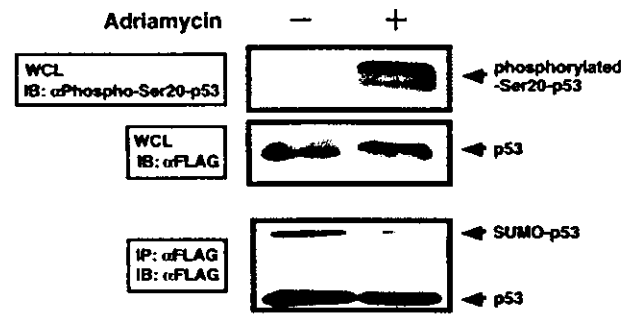


Fig. 3. Sumoylation of p53 is repressed by adriamycin treatment. HEK-293T cells were transfected with 5 μg of plasmid expressing FLAG-tagged wild-type p53. Twenty-four hours after transfection, cells were treated with (+) or without (-) 0.64 μM adriamycin for 24 h, and cell lysates were prepared and IP with anti-FLAG mouse monoclonal antibody. The immunoprecipitates as well as the whole cell lysates were subjected to 7.5% SDS-PAGE and then analyzed by IB using anti-FLAG rabbit polyclonal antiserum.

suggesting that it was repressed by Chk2-mediated phosphorylation of p53 at Ser-20.

It seems that Mdm2 binding to p53 is important for the enhancement of SUMO conjugation to p53 in cells. Since Mdm2 associates with Ubc9, it is possible that Mdm2 enhances the recruitment of Ubc9 to p53. However, this is less likely because sumoylation of p53 in vitro in the presence of Ubc9 was not enhanced by Mdm2. We observed a direct interaction of Ubc9 with p53 in vitro and this interaction was affected by the phosphorylation state of p53. Considering these results and previous reports, it is likely that Ubc9 directly associates and functions to sumoylate p53. Mdm2 may regulate

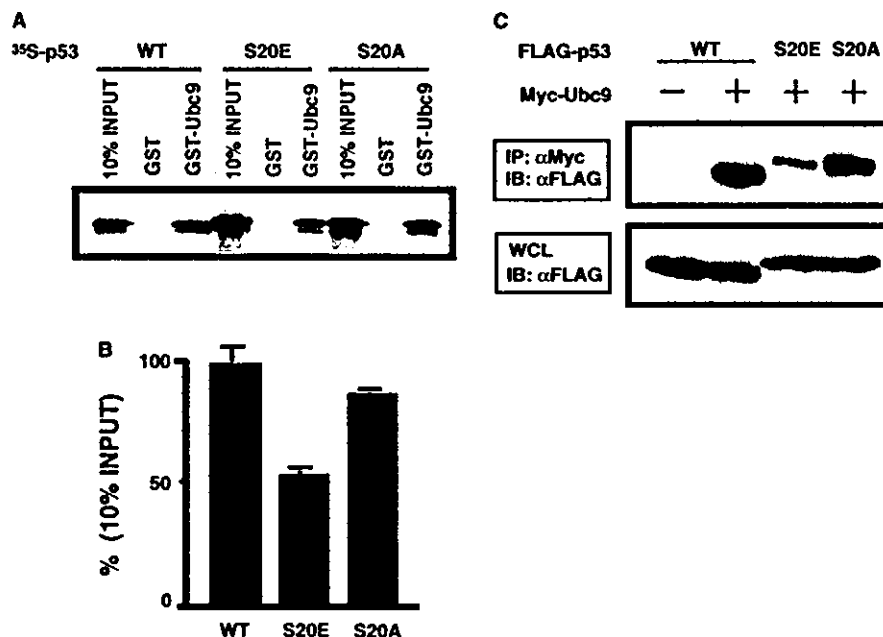


Fig. 2. Association of Ubc9 with wild-type and mutant p53 in vitro and in vivo. (A) ³⁵S-labeled wild-type p53 and p53 mutants, S20E and S20A, were incubated with GST or GST-Ubc9. (B) The GST pull-down complexes were quantitated by imaging analyzer. The experiment was conducted three times independently and data were shown with error bars. (C) HEK-293T cells were transfected with 2 μg of plasmid expressing FLAG-tagged wild-type p53 (WT), S20E, or S20A mutants together with (+) or without (-) Myc-tagged Ubc9 expression plasmid. Thirty six hours after transfection, cell lysates were prepared and subjected to IP with anti-Myc antibody. The immunoprecipitates and the whole cell lysates were subjected to 7.5% SDS-PAGE followed by IB using anti-FLAG rabbit polyclonal antiserum.

this process through at least two mechanisms, enhanced p53 nuclear localization and a mechanism yet to be clarified.

In the present study, we suggest that phosphorylation of p53 at Ser-20 reduces sumoylation. This may result from either the lack of an interaction of p53 with Mdm2 or reduced affinity of Ubc9 to p53 in vivo. Although the physiological roles of sumoylation of p53 are yet to be fully determined, the reduced sumoylation of p53S20E suggests an intrinsic role of sumoylation upon stress induced conditions including DNA damage.

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Siah-1L, a novel transcript variant belonging to the human Siah family of proteins, regulates β -catenin activity in a p53-dependent manner

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β -Catenin is a potent oncogenic protein whose cytoplasmic accumulation is a frequent event in cancer cells. The level of β -catenin is regulated by two mechanisms: the adenomatous polyposis coli/Axin/glycogen synthase kinase 3 β -dependent degradation pathway and the Siah-1/Siah interacting protein/Ebi-mediated degradation pathway. In this study, we have investigated the functional significance of p53-inducible human Siah-family protein expression in the regulation of β -catenin activity. We show here by reverse-transcriptase polymerase chain reaction that two mRNA transcripts, designated human Siah-1 and Siah-1L, are generated from the human Siah-1 locus. Interestingly, the expression of Siah-1L was upregulated by p53, whereas human Siah-1 expression was constant. Furthermore, introduction of exogenous Siah-1L protein downregulated β -catenin protein and promoted apoptosis induced by anticancer drugs in cancer cells that lack endogenous p53. Thus, Siah-1L represents a new member of the human Siah family that is induced in response to p53 and plays an important role in the regulation of β -catenin activity in tumor cells. These findings also suggest new strategies for restoring tumor suppressive pathways lost in cancer cells that have suffered p53 inactivation.

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Introduction

β -Catenin is a multifunctional cytoplasmic protein, which plays an important role in Wnt signalling pathway and in the maintenance of cell–cell adhesion (Provost and Rimm, 1999; Peifer and Polakis, 2000). It has been demonstrated that cellular β -catenin levels are

regulated by two different mechanisms. The first mechanism involves a phosphorylation-dependent pathway. The current model suggests that the Axin and adenomatous polyposis coli (APC) gene products serve as scaffolds to facilitate the phosphorylation of β -catenin by glycogen synthase kinase 3 β (GSK3 β) (Yost *et al.*, 1996; Hart *et al.*, 1998). Phosphorylated β -catenin is targeted for ubiquitin-mediated degradation. The second mechanism is a phosphorylation-independent pathway, which is initiated by an increase in Siah-family protein expression. Siah-family proteins interact sequentially with Siah interacting protein (SIP), Skp1, and Ebi (Matsuzawa and Reed, 2001). Ebi binds directly to β -catenin and initiates a subsequent proteasome-mediated degradation (Matsuzawa and Reed, 2001).

The Siah-family proteins are the mammalian homologues of the *Drosophila* Sina protein. The Siah-family proteins bind ubiquitin-conjugating enzymes via an N-terminal RING domain and target other proteins for degradation (Hu *et al.*, 1997b). The human Siah-1 and Siah-2 genes encode Sina-like proteins (Hu *et al.*, 1997a). The human Siah-1 gene has two murine homologues, Siah-1a and Siah-1b (Della *et al.*, 1993). The murine Siah-1b was reported to be a direct transcriptional target of p53 (Fiucci *et al.*, 2004). Some studies have also suggested that human Siah-1 may act as a downstream effector of p53 (Hu *et al.*, 1997a; Matsuzawa *et al.*, 1998; Roperch *et al.*, 1999; Relaix *et al.*, 2000; Liu *et al.*, 2001; Maeda *et al.*, 2002). However, the mechanism by which p53 induces human Siah-1 gene is still unclear. Moreover, it is possible to hypothesize that findings in murine may be also found in human Siah-1 gene.

Although aberrant accumulation of β -catenin is a frequent event observed in various human cancers including colorectal, lung, and breast cancers, hepatocellular carcinoma (HCC), and hepatoblastoma (Chung, 2000; Lin *et al.*, 2000; Park *et al.*, 2001; Ueda M *et al.*, 2001; Inagawa *et al.*, 2002), mutation of APC, Axin, or β -catenin is not commonly found in several human cancers including HCC (de La Coste *et al.*, 1998). In fact, it has been reported that the β -catenin and Axin are mutated in only about 10 and 7% of HCC, respectively (Satoh *et al.*, 2000; Devereux *et al.*, 2001; Wong *et al.*,

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2001). These results indicate that the cytoplasmic accumulation of β -catenin is not associated with mutations in β -catenin, Axin, or APC in 35–80% of HCCs. Moreover, aberrant accumulation of β -catenin protein without any evidence of dysfunction in the GSK3 β -mediated degradation pathway is not limited to HCCs (Chung, 2000; Lin *et al.*, 2000; Park *et al.*, 2001; Ueda M *et al.*, 2001; Inagawa *et al.*, 2002). Therefore, it is possible to hypothesize that dysfunction in the Siah-1-mediated degradation pathway may contribute to the accumulation of β -catenin in human cancers.

In this study, we have addressed the mechanism of β -catenin protein regulation in tumor cells by investigating the functional significance of p53-inducible Siah-family protein expression. We demonstrated here a novel Siah-1 variant that is involved in the regulation of β -catenin protein degradation in a p53-dependent manner. Our findings thus provide novel insights into the mechanism of β -catenin accumulation in cancers lacking functional p53.

Results

Siah-1L is upregulated in response to p53 and plays an important role in p53-mediated downregulation of β -catenin

The human Siah-1 gene maps to chromosome 16q12–13 (GeneBank accession number NP_010505.11) and consists of two exons and one long intron (~24 kb) (Figure 1a). Using an RNase protection assay, we have previously identified an alternative human Siah-1 mRNA variant that was induced by p53 (Matsuzawa *et al.*, 1998). This mRNA variant contains an additional in-frame ATG start codon (first ATG) upstream of the original ATG (second ATG) (Figure 1a). Indeed, reverse-transcriptase polymerase chain reaction (RT-PCR) using primers specific for human Siah-1 revealed two different transcripts in MCF7 cells, which have wild-type p53 gene (data not shown). The shorter transcript encoded the human Siah-1 mRNA, while

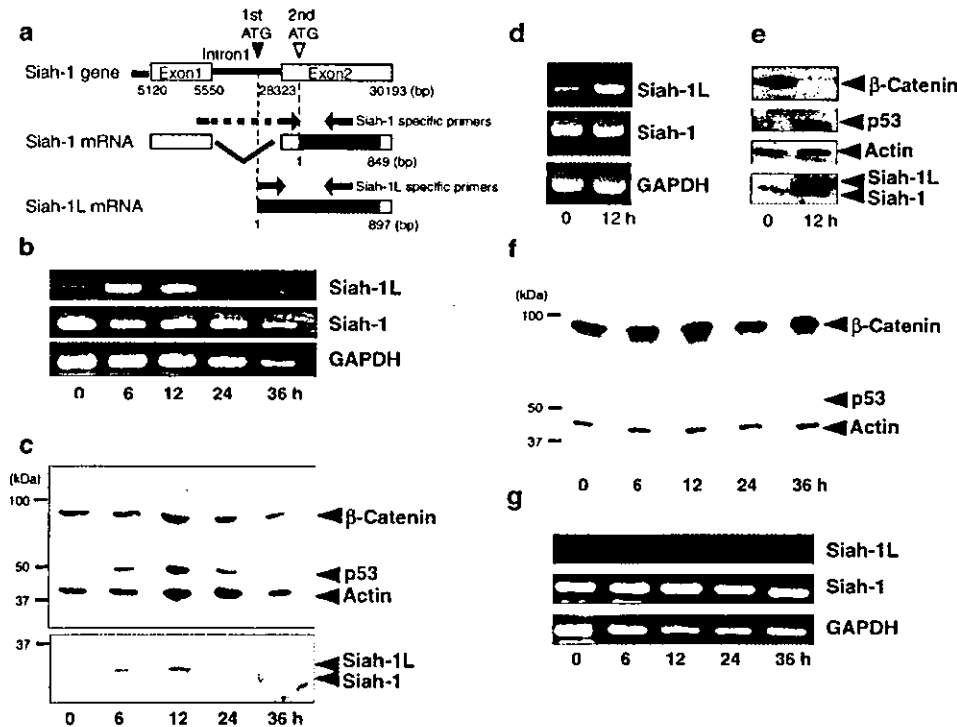


Figure 1 The expression of Siah-1L is induced by p53-mediated signalling. (a) Schematic presentation of the Siah-1L and Siah-1 genes. The Siah-1 gene is shown along with the mRNAs encoding Siah-1L and Siah-1. Splice sites and start and stop positions are shown as lower numbers at their respective genomic nucleotide positions. Ligation of exons 1 and 2 produces the Siah-1 mRNA, whereas inclusion of an extra 48 bp due to an early translation initiation from an ATG codon in the intron produces the Siah-1L mRNA, which encodes a 298-amino-acid protein. The binding sites of the primers used for RT-PCR are outlined. (b) MCF7 cells (p53 wild type) were treated with DOX (1.5 µg/ml). Total RNA was isolated before (0 h) and 6, 12, 24, and 36 h after DOX treatment. RT-PCR was performed using 0.5 µg of each RNA sample as a template and oligonucleotide primer sets specific for Siah-1L (upper panel), Siah-1 (middle panel), and GAPDH (lower panel). (c) Total protein was immediately isolated from MCF7 cells before (0 h) and 6, 12, 24, and 36 h after treatment with DOX (1.5 µg/ml). A 5 µg portion of each protein sample was electrophoresed, blotted, and reacted with antibodies to β -catenin, p53, Siah-1, or α -actin. (d) MCF7 cells were treated with UV irradiation (10 J/m²), and total RNA was isolated immediately before (0 h) and 12 h after UV treatment. RT-PCR was performed using specific primers for Siah-1L (upper panel), Siah-1 (middle panel), and GAPDH (lower panel). (e) MCF7 cells were irradiated at 10 J/m², and total protein was isolated before (0 h) and 12 h after irradiation. A 5 µg portion of each protein sample was analysed by Western blots using anti- β -catenin, anti-p53, anti-Siah-1, or anti- α -actin antibodies. (f) Hep3B (p53-null) cells were harvested before (0 h) and 6, 12, 24, and 36 h after treatment with DOX (1.0 µg/ml). Total protein was isolated and Western blot analyses were carried out using anti- β -catenin, anti-p53, or anti- α -actin. (g) Total RNA was sequentially isolated from Hep3B cells before (0 h) and after DOX treatment (6, 12, 24, and 36 h). RT-PCR was performed using 0.5 µg of each RNA sample and specific primers for Siah-1L, Siah-1, and GAPDH