Tumor Suppressor SMAR1 Activates and Stabilizes p53 through Its Arginine-Serine-rich Motif*

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Various stresses and DNA-damaging agents trigger transcriptional activity of p53 by post-translational modifications, making it a global regulator that controls cell proliferation and apoptosis. Earlier we have shown that the novel MAR-associated protein SMAR1 interacts with p53. Here we delineate the minimal domain of SMAR1 (the arginine-serine-rich domain) that is phosphorylated by protein kinase C family proteins and is responsible for p53 interaction, activation, and stabilization within the nucleus. SMAR1-mediated stabilization of p53 is brought about by inhibiting Mdm2-mediated degradation of p53. We also demonstrate that this arginine-serine (RS)-rich domain triggers the various cell cycle modulating proteins that decide cell fate. Furthermore, phenotypic knock-down experiments using small interfering RNA showed that SMAR1 is required for activation and nuclear retention of p53. The level of phosphorylated p53 was significantly increased in the thymus of SMAR1 transgenic mice, showing in vivo significance of SMAR1 expression. This is the first report that demonstrates the mechanism of action of the MAR-binding protein SMAR1 in modulating the activity of p53, often referred to as the “guardian of the genome.”

The tumor suppressor protein p53 is a short lived, latent transcription factor that is activated and stabilized in response to a wide range of cellular stresses, including DNA damage and activated oncoproteins (1–3). As a potent Cdk inhibitor, p21 is commonly considered to be the major effector of p53-mediated cell cycle arrest (4, 5). Because p21 WAF1 inhibits both the cyclin-dependent G1 kinases and the G2-M-specific cdc2 kinase, p53 is capable of controlling both G1 and G2-M checkpoints (6). Mammalian cell growth is governed by a series of cyclin-dependent kinases (Cdks) whose sequential activation in G1 to S phase and G2-M promotes ordered cell cycle progression (5, 7). The Cdks are regulated through cyclin-subunit interaction, phosphorylation, and by binding of many low molecular weight inhibitor proteins (8–9). One such protein, p21, can inhibit all of these kinases in vitro (5), and its association with inactivated cyclin-Cdk complexes coincides with its ability to invoke G1 and G2-M growth arrest (10–12). The involvement of p53 in DNA damage-dependent delay in the G2 phase of the cell cycle and its contribution to DNA repair in this phase have been described by several studies (13–15). Post-translational modifications of p53 by phosphorylation and acetylation have been proposed to be an important mechanism by which p53 stabilization, as well as its functions, is regulated (16). p53 has been described as a substrate for many kinases in vitro and has been shown to be phosphorylated at a number of serine and threonine residues within the N- and C-terminal regions of the protein (17). There are numerous reports indicating the importance of these post-translational modifications, especially phosphorylation of p53 at various sites in cell cycle regulation (18–20). Various p53-modulating proteins have been identified so far that result in p53 activation in a DNA damage-dependent manner (19, 21). Previously, we have reported another p53-interacting protein, SMAR1 (scaffold/matrix-associated region) (22). SMAR1, a recently identified MARBP, was isolated from double positive mouse thymocytes (23). It specifically binds to a putative MAR (MAR\(\beta\)), a DNase I hypersensitivity site located 400 bp upstream of the transcriptional enhancer (E\(\beta\)) at the T cell receptor \(\beta\) locus (24–25). SMAR1 exists in two alternatively spliced forms: SMAR1\(\alpha\) and SMAR1\(\beta\), with deletion of 39 amino acids in the N terminus in the latter form. The Smar1 gene maps to the distal portion of mouse chromosome 8 at a distance of 111.8 centimorgans (23). Most interestingly, in numerous cancers, altered expressions of several MAR-binding proteins have been demonstrated (26–27). Both wt-p53 and mutant p53 have also been shown to bind to the nuclear matrix (28). However, mutant p53 binds with high affinity to variety of MAR-DNA elements resulting in base unpairing (29–30).

Here we delineate and assign the critical roles of the various domains of SMAR1. Our studies demonstrate that an RRKQR domain along with a stretch of a serine-rich region, encompassing amino acids 160–350 of SMAR1, were responsible for activating p53 mediated by stabilization of the phosphoserine 15 residue of p53 in the absence of any external insults. We demonstrate that the PKC family of proteins played a pivotal role in phosphorylating SMAR1 at its 160–350-amino acid

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domain, which in turn resulted in SMAR1-mediated phosphorylation of p53 at serine 15. The mechanism of SMAR1-mediated p53 stabilization is through displacement of Mdm2 from the p53 N-terminal pocket and hence rescuing p53 from the Mdm2-mediated proteasome degradation. Together these data establish that the SMAR1 is a novel sensor protein that regulates the cell cycle in a mechanism independent of the exog- enously induced DNA damage and through modulation of p53 along with other signal-transducing proteins.

MATERIALS AND METHODS

Plasmid Construction and Transfection—Various truncated forms of SMAR1 were subcloned in-frame with reporter or tag vectors. For 1–245 amino acids, SMAR1 cDNA, full-length constructs from pBK-CMV, was digested with PstI, XbaI, and then digested with EcoRI. An 850-bp fragment corresponding to 1–245 amino acids was then cloned into the EcoRI-Smal site EGF-P-C1 (Clontech). For other truncated mutants like 160–350, 160–400, and 400–548, forward and reverse primers were synthesized with EcoRI and BamHI sites at the 5′ and 3′ ends, respectively. PCR products of respective sizes were first cloned into ToPO-TA cloning vector (Invitrogen). The EcoRI-BamHI fragments from the clonase-derived plasmids were then digested with EcoRI-PstI. For FLAG constructs, EcoRI-HindIII fragments were cloned into 3XFLAG-Neo vector (Sigma). For cloning of 245–288 and 288–350 amino acid regions, the PvuII I site was used which is present at the amino acid 288 junction. EcoRI-PvuII fragment was cloned into EcoRI-Smal of EGF-P-C1. For cloning of the 245–288 region, the insert fragment was isolated by using BamHI-PvuII. EGF-P-C1 vector plasmid was digested with HindIII, made blunt-ended using Klenow and then digested with BamHI. The digested product was then transfected for visualization under the microscope.

For generating the SMAR1-expressing stable line, HK293 cells were transfected by pBK CMV SMAR1 using Lipofectamine 2000. Following transfection, cells were selected by growing in 80 μg/ml G418, and single-cell colony was achieved by limiting dilution.

Western Blotting and Immunoprecipitation—The following antibodies were used: DO-1; anti-p53 (Santa Cruz Biotechnology); anti-FLAG, M2 (Sigma) and anti-SMAR1 (rabbit polyclonal antibody to SMAR1, a kind gift of Dr. M. Tamaru). Cyclins B and E and anti-Mdm2 (Santa Cruz Biotechnology); anti-p53 (Santa Cruz Biotechnology); and 3′-Cy3-conjugated anti-mouse antibody (Banglore Genei) and analyzed by confocal microscopy.

In Vitro Assay of p53 Activation by CAT Assays—K562 cells were seeded at a density of 5 × 10⁶/ml 24 h prior to transfection. Cells were then transfected with different amounts of full-length SMAR1 and various SMAR1 truncations. The CAT assay protocol was followed as described (22). The cells were treated at a density of 5 × 10⁵ cells/ml for 35-mm culture plates. After 24 h, the cells were transfected with Lipofectamine-mediated transfection with either full-length SMAR1 or its truncations expression plasmid DNAs (0.5–1.0 μg); 0.5 μg of pG13CAT; 0.5 μg of p53; 0.2 or 0.5 μg of pGL3 as an internal control. The cells were harvested after 48 h following transfection and lysed in 1× Reporter lysis buffer (Promega). The cells were subjected to three freeze-thaw cycles, followed by centrifugation at 10,000 rpm at 4 °C for 10 min. The supernatant was collected, and the concentrated cell lysates were spectrophotometrically using the Bradford reagent as per the manufacturer’s instructions (Bio-Rad). The CAT activity was assayed by incubating the cell extracts for 4 h at 37 °C in the presence of 1 μCi of [³²P]chloramphenicol (100 μCi/mmol, PerkinElmer Life Sciences) and 100 μg/ml of acetyl-CoA (Amersham Biosciences) in 0.25% Trit-HCl, pH 7.4, in a total of 100-μl reaction volumes. Adding 1 ml of ethyl acetate stopped the reaction; the samples were dried overnight and resuspended in 25 μl of ethyl acetate. The samples were chromatographed on a thin layer chromatography plate (Whatman, Maidstone, UK) and subjected to PhosphorImager (Bio-Rad) scanning and autoradiography.

In Vitro Kinase Assay—5 × 10⁶ HK293 cells were seeded 24 h prior to harvesting either for preparation of whole cell kinase extracts or treatment with increasing concentration of staurosporine (10–30 μM). After 48 h of treatment, kinase extracts were prepared by using the kinase lysis buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 30 mM Na₂VO₃, 1 mM benzamidine, 2 mM PMSF, 1 mM DTT, 0.25% Nonidet P-40, and propidium iodide mixture). After incubating on ice for 20 min, lysates were spun at 14,000 rpm for 10 min. Following this, supernatants were collected, and the concentrated cell lysates were spectrophotometrically using the Bradford reagent as per the manufacturer’s instructions (Bio-Rad). The CAT activity was assayed by incubating the cell extracts for 4 h at 37 °C in the presence of 1 μCi of [³²P]chloramphenicol (100 μCi/mmol, PerkinElmer Life Sciences) and 100 μg/ml of acetyl-CoA (Amersham Biosciences) in 0.25% Trit-HCl, pH 7.4, in a total of 100-μl reaction volumes. Adding 1 ml of ethyl acetate stopped the reaction; the samples were dried overnight and resuspended in 25 μl of ethyl acetate. The samples were chromatographed on a thin layer chromatography plate (Whatman, Maidstone, UK) and subjected to PhosphorImager (Bio-Rad) scanning and autoradiography.
mixture was incubated at 37 °C for 30 min, and then reaction stopped by adding 2× SDS dye. Samples were then run on 8% SDS-PAGE, dried, or stained for Coomassie.

**Generation and Analysis of Transgenic Mice**—For generating SMAR1 transgenic mice, the SMAR1 expressing vector, pBK-CMV-SMAR1, was used where SMAR1 is under the control of the CMV promoter. The construct was first linearized with MluI and ApaLI, giving rise to a 3.8-kb fragment that contains the CMV-promoter along with 1.8-kb SMAR1. The insert was purified on agarose gel, followed by DNA extraction with phenol/chloroform and ethanol precipitation. The DNA pellet was resuspended at a final concentration of 4 μg/ml. DNA microinjection in F2 generation of fertilized (C57BL/6 × SJL) mouse eggs and isolation of tail DNA was performed following the standard protocol. In brief, 4-week-old B6SJLF1 (C57BL/6 × SJL, F1 hybrid) females were used as donors to produce single-cell embryos. Prior to injection, mice were superovulated with 5 IU of pregnant mare serum gonadotropin (MSG) on day 1, followed by 5 IU of human chronic gonadotropin after 48 h. Superovulated females were then mated with proven B6SJLF1 males to generate fertilized embryos for microinjection in the pronuclei. Oviducts were operated out, and embryos were retrieved in RPMI medium. Cumulus mass was removed by incubation of embryos in 1 mg/ml hyaluronidase. Embryos were thoroughly washed in RPMI medium. Single cell F2 hybrid zygotes derived from the cross of F1 hybrid male and female mice were microinjected with DNA containing SMAR1S sequence. Approximately 1–2 pl of DNA (4 μg/μl) was injected into the male pronucleus using the Nikon micromanipulator. The viable embryos that survived microinjection were surgically transferred into the oviducts of CD1 pseudopregnant recipient female (mated with vasectomized males), which carried them to term. The pups born were analyzed for the Smar1 transgene. Analysis of tail DNA for the presence of intact transgene was performed by Southern analysis using 0.6-kb CMV promoter probe.

**Analysis of Smar1 Transgene by Southern Hybridization**—To identify the presence of SMAR1, tail DNA was prepared from mice following standard protocol. Briefly, a 2-mm tail biopsy was incubated in high salt digestion buffer containing 50 mM Tris-HCl, 100 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K for 16 h at 55 °C. The lysates were processed for DNA isolation by phenol/chloroform extraction followed by ethanol precipitation. For Southern analysis, 15–20 μg of DNA was subjected to restriction digestion with BamHI-HindIII enzymes. The digested DNAs were fractionated on a 0.9% agarose gel, followed by denaturation and neutralization. DNA was transferred to Zeta probe membrane. Filters were washed twice for 10 min in 2× SSC and 0.1% SDS at 65 °C. Hybridization signals were detected by PhosphorImaging and autoradiography.

**Detection of Smar1 Transgene by RT-PCR Analysis**—The Smar1 transgene was detected by performing RT-PCR on total cell lysates from thymus transgenic mice as well as the littermate control. RT-PCR assays were done as described previously (22) except that the cDNA was amplified for 27–30 cycles (94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min). RT-PCR products were then separated on a 1.2% agarose gel and visualized by staining with ethidium bromide. Band intensities corresponding to the RT-PCR products were quantified using a PhosphorImager (Bio-Rad) and normalized with respect to the β-actin product.

**Immunohistochemistry**—The thymus was removed and embedded in paraffin, and consecutive sections (3–5 μm) were mounted on slides. Following rehydration and antigen retrieval, sections were immunostained for either p53 or phosphoserine 15 p53 with their respective antibodies (p53 DO-1 and phosphoserine 15 p53, Santa Cruz Biotechnology) and processed for immunohistochemistry with LSAB 2 substrate kit (Dako Corp.) using 3,3′-diaminobenzidine as chromagen (Dako Corp.).

**RESULTS**

**Identification of SMAR1 NLS, Motif Analysis**—Earlier we reported a 548-amino acid MAR-binding protein, SMAR1, isolated from DP mouse thymocyte. It was shown that it shares homology with various MAR-binding proteins like Cux/CDP, SATB1, and Bright (23). To delineate various functional domains of the SMAR1, we made its various truncations and cloned them upstream of GFP. The truncations along with full-length SMAR1 were transiently transfected in HEK 293 cells and then studied for their cellular localization. After 48 h of transfection, full-length SMAR1 along with the truncation comprising 160–350 amino acids were localized within the nucleus (Fig. 1B, panels 1 and 2, respectively). However, other truncations from N-terminal or C-terminal regions (amino acids 1–245 or 350–548) failed to localize within the nucleus (Fig. 1B, panels 3 and 4, respectively), indicating that the NLS domain of SMAR1 resides within amino acids 160–350. To specify further the minimal domain of nuclear import of SMAR1, shorter regions of the 160–350-amino acid truncation were checked by confocal microscopy. The NLS domain lies within amino acids 160–288 as the truncation 288–350 showed a diffused cytoplasmic as well as nuclear localization (Fig. 1B, panels 5 and 6, respectively). Cells were counterstained with DAPI to localize the nucleus (Fig. 1A). Data base search showed no significant homology with any of the known NLS domain. Most interestingly, sequence analysis showed that the 288–350-amino acid truncation had an RRKQR domain along with a serine-rich domain downstream of NLS.

**Overexpression of SMAR1 Helps in Stabilization of p53**—It has been shown that genotoxic insults such as UV- and DNA-damaging agents activate p53 by phosphorylation at its N terminus (18), prevents binding of Mdm2 (17, 32), and stabilizes it within the nucleus (20). We reasoned that SMAR1 overexpression might stabilize p53 through direct interaction and prolonged retention in the nucleus. The p53 status was thus checked in HEK 293 cells, stably transfected with full-length SMAR1. Western blot using anti-SMAR1 confirmed the SMAR1 protein expression in the stable line (Fig. 2C). Endogenous p53 protein levels in control as well as SMAR1 stable cell lines were indirectly labeled with FITC and were visualized by confocal microscopy. In the absence of UV irradiation, p53 in normal (control) cells was localized mostly in the cytoplasm (Fig. 2A, panel 1). In contrast, in the SMAR1 stable cell line, marginal amounts of p53 could be observed within the nucleus, most of it showing cytoplasmic distribution (Fig. 2A, panel 5), indicating that even in the absence of UV irradiation, SMAR1
SMAR1 Phosphorylates p53 and Control Cell Cycle

expression allows retention of p53 in the nucleus. After 4 h of initial UV light exposure (50 J/m²), p53 was observed in both cellular compartments in control cells but was predominantly in the nucleus in SMAR1-expressing cells (Fig. 2A, panels 2 and 6). Most of the p53 in control cells showed cytoplasmic localization at 24 and 36 h, indicating that nuclear p53 was translocated to the cytoplasm (Fig. 2A, panels 3 and 4). Most interestingly, in SMAR1 stable cells, p53 continued to reside within the nucleus for more than 36 h (Fig. 2, A, panels 7 and 8, and B, panels 9 and 10, showing the magnified view of the inset in panels 4 and 8, respectively). Thus, SMAR1 overexpression resulted in accumulation of p53 within the nucleus. To further confirm SMAR1-mediated p53 stabilization, nuclear extracts of UV-treated cells were immunoblotted with antibodies specific for total p53 (Fig. 2D). Prior to UV light exposure, very small amounts of p53 were observed in the nucleus in control (C) or SMAR1 stable cells (S) (Fig. 2D, lanes 1 and 2). After 4 h of UV light exposure, there was a significant increase in total p53. We observed two bands of varying electrophoretic mobility; both lower and upper forms of the protein showed an increase (Fig. 2D, lanes 3 and 4). To verify that the slower mobility band corresponded to the phosphorylated form of p53, the membrane was stripped and reprobed with a phosphoserine 15-specific p53 antibody (Fig. 2E). The phospho-specific antibody recognized only the upper form of p53, confirming that the slow migrating p53 band corresponded to the phosphorylated p53 at serine 15 residue (Fig. 2E). In Fig. 2D, after 24 and 36 h, although there was significant decrease in the phosphorylated p53 in control cells, this was not the case in SMAR1-expressing cells (Fig. 2D, lanes 5–8, respectively). In SMAR1-expressing cells, 2.5- and 4-fold excess p53 was found to be phosphorylated at the 24- and 36-h time points when compared with the normal (control) cells, respectively. Upon reprobing the membrane with phosphoserine 15 p53 antibody, a trace amount of this phosphorylated form of p53 was detected at early time points (Fig. 2E, lanes 1–5 and 7), whereas SMAR1-overexpressing cells showed increased accumulation of the phosphorylated form at later time points (Fig. 2E, lanes 6 and 8). Thus, the MAR-binding nuclear protein SMAR1 allowed stabilization of p53 by enhancing its phosphorylation at serine 15 residue.

p53 Interacting Domain of SMAR1—To analyze the specific domain of SMAR1 that was responsible for its interaction with p53, we have chosen the MDA-MB231 human breast cancer cell line in which p53 remains in the nucleus in the absence of external stimuli. Colocalization studies by confocal microscopy were performed in this line upon overexpression of different SMAR1 truncations. Cells were transiently transfected with expression plasmids encoding either full-length SMAR1 or its truncations as follows: GFP-tagged 1–506, 1–245, 160–350 (D), 400–548 (E), or 350–548 truncation (F). Cells were stained indirectly for p53 using Cy-3-conjugated mouse immunoglobulin (Banglore Genei, India).
were immunoprecipitated (IP) using p53 antibody, and the blot was probed with anti-SMAR1. B, immunoprecipitation was also performed using SMAR1 antibody, and the blot was probed with anti-p53. C, Western blot using anti-FLAG demonstrates the protein expression levels of overexpressed FLAG-tagged full-length SMAR1 along with its truncations in HEK 293 cells. D, lysates from HEK 293 cells transfected with FLAG vector (lane 4), and FLAG-tagged SMAR1 truncations 160–350 (lanes 3 and 5), 400–548 (lane 6), or 1–245 (lane 7) were immunoprecipitated with p53 Ab5 (Oncogene) antibody and probed with 3× FLAG. Lanes 1–3 represent the respective FLAG-tagged SMAR1 truncations, E, anti-FLAG immunoprecipitates were recovered and resolved by SDS-PAGE, and immunoblots were probed by anti-p53 Ab6 (lanes 2–4). The input control for p53 was shown as lane 1.

The region responsible for increased p53 phosphorylation was further mapped to 288–350 residues of SMAR1 (Fig. 5B, lane 4). A region from 160 to 288, which has the NLS, did not show an increase in p53 phosphorylation (Fig. 5B, lane 5). The 288–350 truncated version of SMAR1 is rich in arginine and serine (RS domain) amino acids (Fig. 5A). The RRKQR basic domain and serine-rich domain are both unique in the SMAR1 protein. Thus, a small RS domain of SMAR1 can potentially modulate p53 activity by mediating/regulating its phosphorylation at Ser-15 residue. However, maximum phosphorylation as well as stabilization of p53 occurs when an extended region from 160 to 350 is present. To find out if 160–350 could mediate phosphorylation of other residues of p53, the same filter was reprobed with antibodies specific for p53 S20 (lanes 9, 20, 33, 46), and p53 S392 (lanes 21, 22). No difference in phosphorylation status was observed (data not shown), indicating that 160–350 containing NLS and the arginine-serine rich motif is thus critical for SMAR1-mediated modulation in p53 activity.

Additionally, p53 point mutants namely p53 S15A (serine 15 residue was replaced with alanine) and p53 S20A (serine 20 residue was replaced with alanine) were cotransfected along with FLAG-tagged full-length SMAR1 in p53−/− K562 cells. As a positive control, wild type p53 was cotransfected with FLAG-tagged full-length SMAR1.

**FIG. 4.** SMAR1 directly interacts with p53 through its 160–350 domain. A, a physical interaction of SMAR1 with p53 at endogenous levels was monitored by co-immunoprecipitation assay. HEK 293 cells were immunoprecipitated (IP) using p53 antibody, and the blot was probed with anti-SMAR1. B, immunoprecipitation was also performed using SMAR1 antibody, and the blot was probed with anti-p53. C, Western blot using anti-FLAG demonstrates the protein expression levels of overexpressed FLAG-tagged full-length SMAR1 along with its truncations in HEK 293 cells. D, lysates from HEK 293 cells transfected with FLAG vector (lane 4), and FLAG-tagged SMAR1 truncations 160–350 (lanes 3 and 5), 400–548 (lane 6), or 1–245 (lane 7) were immunoprecipitated with p53 Ab5 (Oncogene) antibody and probed with 3× FLAG. Lanes 1–3 represent the respective FLAG-tagged SMAR1 truncations, E, anti-FLAG immunoprecipitates were recovered and resolved by SDS-PAGE, and immunoblots were probed by anti-p53 Ab6 (lanes 2–4). The input control for p53 was shown as lane 1.

**FIG. 5.** SMAR1 mediates p53 phosphorylation specifically at serine 15 residue. A, physical map of SMAR1 domains. The arginine-serine-rich domain resides within 288–350 amino acids. The black bar shows the location of NLS adjacent to the RS domain. B, lysates from HEK 293 cells transfected with SMAR1 and its truncations were immunoprecipitated with anti-p53 (DO-1) (upper panel), anti-p53 serine 15 (middle panel), or anti-actin as a loading control (lower panel). C, wild type and p53 mutants were cotransfected along with FLAG-tagged full-length SMAR1 in p53−/− K562 cells. Lysates were then immunoblotted with total p53, phospho-p53 serine 15, and 3× FLAG antibodies. ERK was used as the loading control. D, SMAR1 mediates p53 phosphorylation at serine 15 residue in the absence of UV light. Lysates of HEK 293 cells transfected with SMAR1 untreated (lane 1) or treated with UV light (lane 2) (50 J/m2) were immunoprecipitated with anti-FLAG antibodies and then immunoblotted with phospho-p53 serine 15 antibody. E, SMAR1 protects p53 from Mdm2-mediated degradation. HEK 293 cells were transiently transfected with increasing amounts of Mdm2 (0.25–2 μg), and their protein expression was checked by immunoblotting with Mdm2 antibody (upper panel). The status of p53 was then detected using anti-p53 DO-1 (middle panel) and actin (lower panel). F, 5×10^5 HEK 293 cells were transiently co-transfected with increasing amounts of Mdm2 (0.25–2 μg) and SMAR1 (0.5–2 μg). Immunoblotting then checked the expression levels of Mdm2, SMAR1, and p53 with their respective antibodies. Actin was used as the internal control.

Serine 15 of p53 Is the Major Target of Modulation by SMAR1—The p53 protein is known to interact with numerous other proteins (2) and is subject to several post-translational modifications (18, 20). Lately, it is beginning to understand how these protein interactions and modifications contribute to the regulation of p53 stability and activity. On the basis of the observation that an interaction between SMAR1 and p53 results in the stabilization of p53 within the nucleus, the p53 phosphorylation status was thus examined. Whole cell lysates of HEK 293 cells transiently transfected with various GFP-tagged truncations of SMAR1 were immunoblotted with antibodies that detect total or phosphoserine 15 p53. Although there was no significant difference in the total p53 protein level (Fig. 5B), increased p53 phosphorylation was observed in cells expressing either full-length or the 160–350 truncation of SMAR1 (2.3- and 3-fold respectively) (Fig. 5B, lanes 2 and 3).
the cells were lysed and immunoblotted with p53 (DO-1), phospho-p53 Ser-15, and 3× FLAG antibodies. As expected, the SMAR1 was unable to phosphorylate p53 S15A mutant but successfully phosphorylated both wild type p53 and p53 S20A in p53−/− K562 cells (Fig. 5C, lane 3 and lanes 1 and 2, respectively). ERK was used as the loading control, whereas equal SMAR1 expression was also confirmed using anti-FLAG. This strongly suggested that the SMAR1 mediated phosphorylation of the serine 15 residue of p53.

To test further whether stabilization of phosphorylated p53 (p53 serine 15) was due to its interaction with SMAR1 and not because of exogenous insult like UV light, cells were transiently transfected with FLAG-tagged SMAR1. Whole cell lysates from UV-treated or control (untreated) cells were then immunoprecipitated with FLAG antibody followed by immunoblotting with antibodies to phospho-p53-serine 15. In both cases, phospho-Ser-15 p53 was immunoprecipitated to equivalent levels (Fig. 5D, lanes 1 and 2), indicating that SMAR1 alone allowed stabilization of p53 serine 15 in the absence of UV damage.

**SMAR1 Protects p53 from Mdm2-mediated Degradation**

The intracellular activity and stabilization of p53 is regulated through a feedback loop involving its transcriptional target Mdm2 (32–34). Numerous reports have demonstrated that p53 stabilization is brought either by induction of covalent modifications in Mdm2 and p53 or through altered protein-protein interactions (33–34). In order to understand the mechanism of action of SMAR1-mediated p53 stabilization, experiments were performed to determine the effect of Mdm2 over p53 degradation in the presence of SMAR1. Upon transient transfection of increasing amounts of Mdm2 (0.25–2 μg) in HEK 293 cells, we observed that there was increased Mdm2-mediated p53 degradation. A dose-dependent decrease in the p53 protein expression was observed as the amount of Mdm2 was increased (Fig. 5E, lanes 2–5). However, upon cotransfection of Mdm2 along with varying amounts of SMAR1 (0.5–2 μg), an increase in the p53 protein levels was observed (Fig. 5F, lanes 3–5). At the highest concentration (2 μg) of co-expression of SMAR1 and Mdm2 in HEK 293 cells, there was more than 2-fold increase in p53 expression when compared with Mdm2 alone (Fig. 5, F, lane 5, and E, lane 5, respectively). Thus, in presence of SMAR1, overexpression of Mdm2 was ineffective in causing p53 degradation (Fig. 5F, lane 5), suggesting that SMAR1 rescues p53 from Mdm2 and thus does not allow Mdm2 to bind and export p53 out of the nucleus for proteasome degradation. Actin was used as an internal control (Fig. 5, E and F).

**Arginine-Serine-rich Minimal Domain of SMAR1 Activates p53**—To evaluate SMAR1-mediated modification and functional activation of p53, CAT reporter assays were performed following expression of full-length or truncations of SMAR1. The SMAR1 GFP-tagged truncations were cotransfected with p53 reporter plasmid PG13 in K562 cells (p53−/−) cells, and their protein expression levels were confirmed by using anti-GFP in HEK 293 cells ectopically expressing SMAR1 and its truncations were immunoblotted with antibodies to p21, Mdm2, phospho-cdc2, and total cdc-2 (Fig. 7A). Both p21 and Mdm2 were up-regulated upon expression of either the full-length or the p53-activating domain of SMAR1 (Fig. 7A, lanes 2, 4, and 5). In contrast, the truncation consisting of amino acids 160–288 did not show any up-regulation of either p21 or Mdm2 levels (Fig. 7A, lane 3). The level of the cdc2 is critical for the regulation of the cell cycle, and any dysregulation sensed by the cell results in triggering of cascades leading to either mitotic arrest or apoptosis (40). Upon the overexpression of SMAR1, there was 2.8-fold increase of phospho-cdc2 (Fig. 7A, lane 2). Although SMAR1 region 160–350 increased phospho-cdc2 levels about 8.5-fold (Fig. 7A, lane 4), the region 160–288 did not show any significant increase (Fig. 7A, lane 3). The smallest truncation encompassing amino acids 288–350, containing the RS domain increased p53 activity about 3.5–4-fold (Fig. 6, B, lane 9, and C, bar 7). Thus, the p53 modulating activity of SMAR1 lies within this short region containing the RS domain.

**Modulation of the p53-mediated Downstream Signaling Cascade by SMAR1**—To understand the significance of SMAR1-mediated p53 activation, the expression of several signal-transducing proteins controlling cellular proliferation was studied. Phosphorylation of p53 dissociates it from Mdm2, activating its DNA binding activity (17, 32, 34). One of the genes activated by p53 is p21 Cip1, an inhibitor of a subset of the cyclin-dependent kinases including cdc2 (5, 11, 39). To find out if SMAR1 altered the expression of these effector molecules, whole cell lysates of HEK 293 cells ectopically expressing SMAR1 and its truncations were immunoblotted with antibodies to cdc2 (Fig. 7A). Both p21 and Mdm2 were up-regulated upon expression of either the full-length or the p53-activating domain of SMAR1 (Fig. 7A, lanes 2, 4, and 5). In contrast, the truncation consisting of amino acids 160–288 did not show any up-regulation of either p21 or Mdm2 levels (Fig. 7A, lane 3). The level of the cdc2 is critical for the regulation of the cell cycle, and any dysregulation sensed by the cell results in triggering of cascades leading to either mitotic arrest or apoptosis (40). Upon the overexpression of SMAR1, there was 2.8-fold increase of phospho-cdc2 (Fig. 7A, lane 2). Although SMAR1 region 160–350 increased phospho-cdc2 levels about 8.5-fold (Fig. 7A, lane 4), the region 160–288 did not show any significant increase (Fig. 7A, lane 3). The smallest truncation encompassing amino acids 288–350, containing the RS do-
main, also increased the expression of phospho-cdc2 comparable with the 160–350 truncation (Fig. 7A, lane 5). However, no difference in total cdc-2 levels was observed (Fig. 7A, lanes 1–5). These results indicate that SMAR1 through its RS domain cooperates and activates p53 that in turn modulates the downstream cell cycle regulatory proteins.

**SMAR1 Alters the Expression of Mitogen-activated Protein Kinase**—To analyze further the effect of SMAR1 and its truncations on other cell cycle regulatory cascades, lysates from HEK 293 cells expressing full-length or various truncated forms of SMAR1 were probed with specific antibodies as shown in Fig. 7B. There was a 1.6–2-fold down-regulation of phospho-ERK (mitogen-activated protein kinase) in lysates from cell expressing full-length, 160–400, 160–350, or 288–350 truncated forms of SMAR1 (Fig. 7B, lanes 3–6); the N-terminal end of SMAR1, amino acids 1–245, did not cause this decrease in phospho-ERK levels (Fig. 7B, lane 2). Under identical conditions, overexpression of either the full-length or truncated SMAR1 proteins did not result in changes in the total ERK levels, showing that SMAR1 and its minimal RS region are responsible for ERK inactivation. No changes were observed in the levels of cyclin B, cyclin E, or AKT in these cells (Fig. 7B, lanes 1–5).

**PKC Family of Proteins Phosphorylates SMAR1**—As we had demonstrated that SMAR1 could mediate p53 phosphorylation specifically at the serine 15 residue, the mechanism of SMAR1 phosphorylation was still unclear. We performed an in vitro phosphorylation assay by using either full-length or all truncated domains of SMAR1 with whole cell kinase extracts prepared from HEK 293 cells. Both the full-length and the region 160–350 (containing RS domain) were phosphorylated as shown by a shift in the mobility of the band in SDS-PAGE (Fig. 8A, upper panel). The regions that failed to phosphorylate were 350–548 and 400–548 (Fig. 8A, lower panel). In a similar approach, an increasing amount of purified GST-SMAR1 (1–2 μg) was checked for [γ-32P]ATP incorporation after setting an in vitro kinase reaction using whole cellular kinase extracts from HEK 293 cells (Fig. 8B). We observed that SMAR1 was efficiently phosphorylated. An immunoblot with phosphoserine-specific antibody further confirmed that SMAR1 was phosphorylated at the serine residue in the presence of whole cell kinases (Fig. 8C), whereas GST-SMAR1 alone was unable to phosphorylate, indicating that SMAR1 cannot be autophosphorylated. As we observed that only the 160–350 region of SMAR1 could be phosphorylated and to further understand the mechanism or the kinase, which could be responsible for causing post-translational modification of SMAR1, we scanned the sequence of the 160–350 region of SMAR1, and most interestingly, we found that this region is composed of the substrate phosphorylation motif for the PKC family (35, 36) (Fig. 8D). Fig. 8D depicts the sequence of the PKC substrate motif in the SMAR1 peptide along with its serine mutant counterpart. To confirm the computational data, we performed in vitro kinase assays using GST-treated kinase lysates (37, 38). The SMAR1 phosphorylation status was then correlated to [γ-32P]ATP incorporation. We observed that upon staurosporine treatment, the phosphorylation of SMAR1 is almost inhibited as shown in Fig. 8E, lanes 2 and 3. Furthermore, an immunoblot with p53 serine 15 antibody demonstrated that SMAR1 was unable to phosphorylate p53 when HEK 293 cells were treated with staurosporine in increasing concentrations (10–30 nM) (Fig. 8F, lower panel). The inhibition of SMAR1-mediated p53 phosphorylation upon staurosporine treatment strongly suggested a critical role of PKC family in SMAR1 phosphorylation, which in turn phosphorylates p53. This suggests that the PKC family phosphorylates SMAR1 which in turn allows p53 phosphorylation.

**Knock-down of SMAR1 Using siRNA**—Numerous studies imply the existence of multiple pathways involved in p53 stabilization (20, 31). In order to address the role of SMAR1 in p53 activation and henceforth stabilization, we designed an siRNA corresponding to the arginine-serine-rich (the RS domain) sequence of SMAR1 (SMAR1 siRNA). A scrambled sequence was used as a negative control (Scr). A BLAST search indicated that our SMAR1 siRNA sequence is restricted to SMAR1, and the scrambled siRNA sequence is absent altogether. The siRNA was cotransfected along with the SMAR1 (FLAG- or GFP-tagged) construct into HEK 293 cells in a dose-dependent manner as mentioned under “Materials and Methods,” and protein expression levels were detected 48 h post-transfection. Transient expression of 100–200 nM of SMAR1 siRNA almost completely inhibited the expression of both FLAG-tagged (Fig. 9A) as well as GFP-tagged SMAR1 (Fig. 9B). As expected, no difference in the SMAR1 protein levels was observed when cells
were transfected with the scrambled siRNA (Fig. 9C). Silencing of the Smar1 genes was also confirmed by inhibition of the expression of corresponding mRNAs using RT-PCR (Fig. 9D). The effect of this siRNA was also checked on other MAR-binding proteins like Cux/CDP. There was no difference in the protein expression levels of either of the MARBPs upon siRNA treatment (Fig. 9E).

Once established, we used these siRNAs to investigate the role of SMAR1 in p53 phosphorylation and its stabilization. The HEK 293 cells were cotransfected with FLAG-tagged SMAR1 and Scrambled or SMAR1 siRNAs and then monitored for the expression of phosphorylated p53 serine 15. Immunoblotting with anti-p53 showed that inhibition of SMAR1 by siRNA significantly affected the expression of activated p53 as observed through gradual loss of the phosphorylated band (Fig. 9F, upper panel, lanes 5–7). Immunoblotting of the same siRNA-treated protein lysates with anti-serine 15 phospho-p53 further emphasized the effect of SMAR1 inhibition on p53 phosphorylation and hence stabilization.

For p53 expression in SMAR1 transgenic mice, SMAR1 was identified from mouse thymocytes and was found to be overexpressed in double positive stage of thymocyte development (23).

Upon restriction enzyme digestion and Southern blot analysis, 0.7-kb CMV promoter along with the 2.1-kb SMAR1 gene was identified from mouse thymocytes and was found to be overexpressed in double positive stage of thymocyte development (23). To generate SMAR1 transgenic mouse, as detailed under “Materials and Methods,” the 2.8-kb DNA fragment containing the 0.7-kb CMV promoter along with the 2.1-kb SMAR1 gene was injected into the pronucleus. The transgene was checked in the thymus by using the 0.6-kb CMV promoter probe (Fig. 10A). Upon restriction enzyme digestion and Southern blot analysis,
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FIG. 10. p53 is overexpressed in SMAR1 transgenic mouse thymus. A, map of 2.8-kb DNA fragment containing 0.7-kb CMV promoter along with 2.1-kb Smar1 gene that was injected into the pronucleus of mice to generate SMAR1 transgene. Restriction enzyme sites along with their respective sizes are depicted. B, Southern blot analysis to check the SMAR1 transgene using 0.6-kb CMV promoter probe. C, RT-PCR analysis of SMAR1 mRNA expression from the tissue of normal littermate (LM) versus SMAR1 transgenic (Tg). β-Actin and SMAR1 indicate the position of the respective expected RT-PCR products. D, tissues were isolated from normal (N), normal littermate (LM), and SMAR1 transgenic mice (Tg), and the lysates were immunoblotted with total p53, anti-phospho-p53 serine 15, and SMAR1 antibody. Total ERK was used as an internal control. E and F, immunostaining of thymus tissue from normal littermate (LM) and transgenic mice (Tg). Left panels show hematoxylin and eosin (HE) staining, although middle and right panels correspond to staining with total p53 and anti-phospho-p53 serine 15 followed by detection by dianobenidine, respectively.

a 3.8-kb fragment was found to be hybridized in the case of transgenic mice (Fig. 10B, lane 2). The level of transgene expression was further confirmed by RT-PCR analysis using primers specific for SMAR1 in both littermate (negative for the SMAR1 transgene) and the SMAR1 transgenic mice (Fig. 10C). β-Actin was used as the loading control. To find the p53 expression in transgenic mice (Tg), Western blot were performed either anti-p53, phospho-p53 serine 15, or SMAR1 antibody and compared with normal along with normal littermate mice. Most interestingly, in transgene-positive mice, there was a strong up-regulation of SMAR1 expression in thymocytes along with total p53 (Fig. 10D). There was 4–6-fold up-regulation of SMAR1 and p53 serine 15 was observed in transgenic mice compared with either normal or littermate control mice (Fig. 10D, lane 3 compared with lanes 1 and 2, respectively). We also observed that the number of thymocytes was lowered by 1.8-fold in transgenic mice compared with that of littermate normal mice suggesting that p53 might be responsible for perturbed cell cycle pathway of thymocytes.2 Thus, overexpression of SMAR1 causes up-regulation of total p53 in transgenic mice. On immunostaining of histology sections of thymus from both littermate and the transgenic with anti-p53 phospho-Ser-15 or total p53, we observed significantly higher expression of both total p53 as well as phospho-p53 (serine 15) in transgenic mice (Fig. 10, E and F, respectively). The strong up-regulation of p53 in SMAR1 transgenic mice thus correlates with the in vitro experiments and further confirms that SMAR1 overexpression results in the higher p53 phosphorylation both in vitro as well as in vivo.

DISCUSSION

MARs and MAR-binding proteins play an important role in chromatin modulation and transcriptional control (41). SMAR1, a newly identified MAR-binding protein involved in chromatin-mediated gene regulation, exists in two alternatively spliced forms (23). Recently we have also shown it to interact with another MAR-binding protein CDP/Cux and thus regulate the T cell receptor β enhancer unraveling its pivotal role in V(D)J recombination (42). In our earlier work we have shown that SMAR1 regulates the cell cycle via its interaction with p53 (22). Here we have delineated the nuclear localization signal as well as the functional domain of SMAR1 encompassing amino acids 160–350. This domain consists of five consecutive serine residues flanked by an arginine-rich motif (RS domain). We also demonstrate that this RS domain, amino acids 160–350, is the minimal domain of SMAR1 that interacts with p53 and modulates its activity through phosphorylation at the serine 15 residue. Serine 15 phosphorylation of p53 is associated with increased transcription efficiency, decreased affinity for Mdm2, and increased nuclear localization (17, 32). From all the previous studies on the control of p53 transcription and translation, it seems evident that the major mechanisms controlling p53 function are as follows: regulation of p53 protein levels, control of the localization of the p53 protein, and modulation of the activity of p53. Here we also demonstrate that PKC family of serine-threonine kinases can bring about post-translational modification of SMAR1 through phosphorylation at a serine residue present within the 160–350 region. It has also been shown that wild type p53 is associated with the nuclear matrix (28). It is possible that SMAR1, upon phosphorylation, although being associated with the nuclear matrix simultaneously anchors p53 and activates it by mediating phosphorylation at its N terminus. Using CAT reporter gene assays, we herein demonstrate that the minimal domain (amino acids 160–350) of SMAR1 was sufficient to activate p53. Furthermore, phenotypic knockdown experiments using SMAR1 siRNA demonstrate that SMAR1 played a critical role in regulating p53 activation mediated via stabilization of p53 specifically of the phosphoserine 15 residue. This silencing of SMAR1 destabilized p53 and induced it to undergo Mdm2-mediated proteasomal degradation. Multiple lines of evidence indicate that the lion’s share of the negative regulation of p53 is performed by the Mdm2 protein (32, 34). The inactivation of p53 by Mdm2 is achieved through two distinct molecular mechanisms. On the one hand, Mdm2 binds tightly to the N terminus transactivation domain of p53 and thus blocks critical interactions with other proteins necessary for p53-dependent regulation of gene expression. On the other hand, Mdm2 plays a crucial role in the proteasome-mediated p53 degradation (32–34). Here we demonstrate that SMAR1 was able to rescue/protect p53 against Mdm2-mediated degradation. These observations suggest that phosphorylation of p53 at its serine 15 residue mediated through SMAR1 may augment nuclear retention of p53 in two ways, first by directly inhibiting the N-terminal nuclear export sequence (43), and second by inhibiting Mdm2 binding and in turn preventing Mdm2-mediated degradation (20, 44). Most interestingly, the minimal RS domain of SMAR1 was sufficient in up-regulating the tyrosine 15 phosphorylation of cdc2, a key regulator of the G1-M transition, indicating that the region is critical for mediating the functions of SMAR1.
of SMAR1. Cdc2-Tyr-15 dephosphorylation and mitosis are inhibited in response to the activation of the DNA replication and damage checkpoint (45–46), further confirming its role in cell cycle regulation. FACT, a partner in small nuclear ribonucleoprotein complex, is also shown to interact with p53 and retinoblastoma through its serine-arginine motif (47). Also, such an RS domain has been shown earlier to allow formation of the splicing complex (48). The RS domain of SMAR1 plays a special role in controlling the cell cycle through modification of p53 at the serine 15 residue. Both serine 15 and serine 20 are major phosphorylation targets following various forms of p53 activation that may link the p53 response to other aspects of cellular metabolism. The SMAR1 modification pathway may therefore represent a novel target for the development of therapeutically useful modulators of the p53 response.

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