Osmotic shock induces G1-arrest through p53 phosphorylation at ser33 by activated p38MAPK without phosphorylation at ser15 and ser20

Hiroto Kishi¹,², Kazumi Nakagawa¹, Mitsuhiro Matsumoto², Moritaka Suga², Masayuki Ando², Yoichi Taya³, and Masaru Yamaizumi*¹

¹Institute of Molecular Embryology and Genetics, Kumamoto University, Kuhonji 4-24-1, Kumamoto 862-0976, Japan

²First Department of Internal Medicine, Kumamoto University School of Medicine, Honjo 1-1-1, Kumamoto 860-0811, Japan

³Radiobiology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan

Running title: Activation of p53 by p38MAPK following osmotic shock

*Corresponding author.
Telephone: 81-96-373-6603  
FAX: 81-96-373-6604  
E-mail: yamaizm@gpo.kumamoto-u.ac.jp
Summary

Osmotic shock induced transient stabilization of p53, possibly due to increased degradation of Mdm2. Stabilized p53 was activated by p38MAPK, resulting in G1-arrest through induction of p21WAF1. Among the postulated phosphorylation sites involved in p53 stabilization or activation (ser15, ser20, ser33, and ser46), only ser33 was phosphorylated. Furthermore, interaction of p53 with the transcriptional coactivator p300 was induced, and lys382 of p53 was acetylated. Although inhibition of p38MAPK did not prevent nuclear accumulation of p53, phosphorylation of ser33 was markedly suppressed by SB203580, a specific inhibitor of p38MAPK. Under these conditions, acetylation of lys382 and induction of p21WAF were also inhibited, and cells with elevated levels of p53 showed normal cell cycle progression. Activated p38MAPK phosphorylated endogenous p53 at ser33 in living cells. In stable transformants expressing dominant negative MKK6, an upstream protein kinase of p38MAPK, p53 stabilization was induced normally following osmotic shock, but phosphorylation of ser33, acetylation of lys382, and induction of p21WAF1 were almost completely inhibited. These results suggest that phosphorylation at ser33 by p38MAPK is critical for activation of p53 following osmotic shock. Phosphorylation of neither ser15 nor ser20 was needed in this activation.
Introduction

Cells respond to environmental stress with multiple defense systems for the maintenance of homeostasis or adaptation. Exposure of cells to hyperosmotic media initiates an immediate response that regulates cell volume. In the yeast *Saccharomyces cerevisiae*, a MAP kinase, Hog1, has been implicated in this response. Activation of Hog1 up-regulates the activity of glycerol-3-phosphate dehydrogenase encoded by the GPD1 gene, which stimulates accumulation of glycerol, thus increasing intracellular osmolarity (1). Mutants defective in Hog1 expression cannot grow in hyperosmotic media (2). In mammalian cells, exposure to hyperosmotic media causes activation of p38MAPK, a homologue of Hog1 (3). While Hog1 is activated only by osmotic stress, p38MAPK is activated by a wide range of stress, such as osmotic shock, UV, heat shock, nutritional starvation, and cytokines. In vertebrates, some tissues such as small intestine (4) and colon (5) are routinely exposed to hyperosmotic tissue fluid, and in renal medulla, the osmolarity of the interstitial fluid of urinary tubules is often more than five times higher than normal (6), suggesting involvement of p38MAPK in the response to osmotic challenge in daily life.

Mammalian cells accumulate and activate the tumor suppressor protein p53 after exposure to genotoxic or environmental stress like heat shock (7-10). Activated p53 protein functions as a transcription factor for different groups of genes involved in the cell cycle checkpoint [p21WAF1 (11)], DNA repair [Gadd45 (12), DDB2 (13), p53R2 (14), etc], or apoptosis [Bax (15), p53AIP1 (16), etc]. Cell cycle arrest is mediated by enhancement of p53-dependent expression of p21WAF1, which is a general inhibitor of
cyclin-dependent protein kinases (Cdks). This checkpoint control enhances genetic fidelity by causing arrest at specific stages of the cell cycle when previous events have not been completed. p53 is a short-lived protein with a half-life of 20-40 min. This is due to its rapid ubiquitination by Mdm2 and subsequent degradation by 26S proteasome (17, 18). In this way, only a trace amount of p53 is detected in rapidly growing normal cells. Recent studies revealed that accumulation of p53 following genotoxic stress is mainly caused by suppression of degradation. Interaction of p53 and Mdm2 is mediated by an N-terminal region of p53 (thr18-lys34) (19). X-ray and UV irradiation somehow activates ATM (12) and ATR (20), respectively. These kinases then activate a down-stream chk1 or chk2 checkpoint kinase which phosphorylates ser20 of p53 (21, 22). p53 phosphorylated at ser20 escapes from degradation through diminished interaction with Mdm2 (23, 24). In cells treated with hypoxia, the Mdm2 protein level is down-regulated, which is a likely mechanism for accumulation of p53 (25, 26). Activation of p53 is also reported to be controlled by phosphorylation of p53 at critical serine residues in the N-terminus. Activated ATM or ATR can phosphorylate ser15 in vivo and in vitro (27-30). Recently, it was reported that UV and chemotherapeutic agents activate p38MAPK, and that activated p38MAPK phosphorylates ser33 plus ser46, and ser33 alone, respectively (31, 32). Another potential mechanism that may play a critical role in p53 activation is acetylation. In an in vitro experiment, lys320 is acetylated by PCAF (33), while lys373 and lys382 are acetylated by p300/CBP (33, 34). Phosphorylation at ser15 stimulates interaction between p53 and its transcriptional coactivators p300/CBP. Substitution of ser15 causes a defect in p53-
dependent transcriptional activation (35, 36). Furthermore, p300/CBP-mediated acetylation of p53 is commonly observed in vivo after treatment of cells with multiple p53-activating agents (37).

Until now, the molecular mechanisms of the stabilization and activation of p53 have been analyzed mainly with cells treated with DNA damaging agents such as ionizing radiation (IR), chemotherapeutic drugs, or UV. However, these agents produce different types of reactive radicals which may damage cellular components along with the DNA. In such cases, multiple signaling pathways might be activated simultaneously, which complicates analysis of the overall response process. Compared with these treatments, osmotic shock and hypoxia seem simple, because they have little effect on DNA. Previously, we reported that nuclear accumulation of p53 is evoked in normal human fibroblasts cultured in hyperosmotic medium (38). Recently it was reported that p53 accumulated under hyperosmotic conditions was transcriptionally active in a murine renal inner medullary collecting duct cell line (mIMCD3) (39). However, little is known about the molecular mechanism of osmotic shock-induced p53 accumulation and its effect on the cell cycle checkpoint. In the present study, we found that p53 was transiently stabilized by hyperosmotic treatment, independently of phosphorylation of ser15 and ser20 in normal human fibroblasts. However, phosphorylation of ser33 is critical for cell cycle arrest through induction of p21<sub>WAF1</sub>, and this phosphorylation is mediated by activated p38<sub>MAPK</sub>. Furthermore, osmotic shock enhances interaction of p53 with p300, resulting in acetylation of lys382 of p53. These results clearly show that osmotic shock induces p53-dependent cell
cycle arrest through activation of p38MAPK together with the immediate adaptation response.
Experimental procedures

Cells and hyperosmotic treatment

Mori (40) is a primary cell strain of normal human fibroblasts. H1299 and A549 are human lung carcinoma cell lines. MCF7 is a human mammary carcinoma cell line. Primary mouse fibroblasts were obtained from embryonic lungs of the C57BL-6J strain, and p53 gene knock-out mice (41). All cells were cultured in Dulbecco's modified Eagle's MEM (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin G (100 U/ml), and streptomycin (100 µg/ml) in a humidified 5% CO₂ incubator. For osmotic shock, Mori, H1299, A549, and primary mouse fibroblasts were treated in hyperosmotic medium containing 240 mM NaCl. This medium was prepared by adding 65 µl of 2 M NaCl to 1.0 ml of culture medium containing 10% FCS. MCF7 cells were treated in hyperosmotic medium containing 260 mM NaCl. p38MAPK kinase inhibitors, SB203580 and SB202190, were purchased from Calbiochem. The concentration of solvent (dimethyl sulfoxide, DMSO) was kept constant at 0.1% in all cultures. To obtain stable transformants, A549 or MCF7 cells were transfected with 10 µg of pcDNA3-HA-MKK6AA or pcDNA3 by electroporation using an Electro Cell Manipulator (BTX, San Diego, CA), and stable transformants were selected with G418 at concentrations of 800 µg/ml and 500 µg/ml, respectively. At least 10 independent clones were isolated. Expression of HA-MKK6AA was checked by indirect immunostaining using a monoclonal antibody against HA (HA. 11, Babco). The clone expressing the highest level of HA was used.
Construction of plasmids

pCS3-Myc-p38^{MAPK}, pME-HA-MKK6AA, and pcDNA3.1(+)-MKK6EE were kindly provided by Y. Gotoh (Univ. of Tokyo). HA-MKK6AA was recloned into pcDNA3 (Invitrogen). p53 cDNA was cloned into the pRc-CMV expression vector. To substitute ser15, ser33, or both with alanine, the Quick Change™ Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions. Replacement at individual sites was confirmed by DNA sequence analysis.

Immunostaining

Mori and mouse primary fibroblasts were cultured on glass-coverslips. The cells were treated for various periods in hyperosmotic media containing 240~260 mM NaCl. In control experiments, cells were irradiated with X-rays, and incubated for various periods at 37°C. These cells were prefixed with 3.7% formaldehyde for 2 min at room temperature, washed with PBS, and then fixed with 80% methanol for 10 min at -10°C. Cells were stained with antibodies against p53 (PAb1801, Calbiochem) for 30 min at room temperature. To detect phosphorylation of specific serine residues, anti p53-P-ser15 (42), anti p53-P-ser33 (43), and anti p53-P-ser46 (16) polyclonal rabbit antibodies were used with non-phospho blocking peptides. After a wash with PBS, the cells were stained with either FITC-conjugated anti-mouse or FITC-conjugated anti-rabbit IgG, or in some cases, Rho-conjugated anti-mouse IgG. For staining of the phosphorylated ser15, 33, or 46 of p53, cells were further stained with FITC-conjugated anti-goat IgG to enhance the intensity of fluorescence (Chemicon Internation Inc.). To detect the HA
tag, monoclonal antibody against HA (HA. 11, Babco) was used as the first antibody.

**Western blotting**

After treatment in the hyperosmotic media, cells were harvested with trypsin-EDTA and washed with PBS. Then, whole cell extracts were prepared in lysis buffer [1.7% sodium dodecyl sulfate, 17% glycerol, 0.1 M dithiothreitol, 0.083 M Tris (pH 6.8)]. p53 protein, p21\(^{\text{WAF1}}\) protein, Mdm2 protein, α-tubulin, and HA-tag were detected by immunoblotting with monoclonal antibodies of PAb1801, OP64 (Oncogene Science), SMP14 (Neo Markers), OP06 (Calbiochem), and HA. 11 (Babco), respectively. Phosphorylated ser15, ser20, ser33, and ser46 of p53 were detected with rabbit polyclonal antibodies against phospho-ser15 (42), phospho-ser20 (44), phospho-ser33, and phospho-ser46, respectively. Acetylated lys382 of p53 was detected with a rabbit polyclonal antibody against acetylated lys382 of p53 (34). p38\(^{\text{MAPK}}\) protein, and p300 protein were detected with rabbit polyclonal antibodies of C-20 (Santa Cruz), and N-15 (Santa Cruz), respectively. Immunoblots were developed with an ECL detection system (Amersham).

**Immunoprecipitation**

Cells (10\(^7\)~10\(^8\)) were lysed in TEG buffer (26), and p300 was immunoprecipitated with an antibody against the protein (N-15, Santa Cruz) and protein G-Sepharose. The Sepharose beads were then washed, and bound proteins were separated by SDS-PAGE and electrophoretically transferred to a PVDF membrane filter. p53 or p300
protein was detected with the antibody DO1 (Calbiochem) or N-15 (Santa Cruz), respectively, using an ECL detection system (Amersham).

**Cell cycle analysis**

Cell cycle analysis was performed as described elsewhere (10). Briefly, cells were cultured in plastic dishes (60 mm in diameter) for at least 2 days. They were then treated in hyperosmotic media for various periods. Before harvest by trypsinization, cells were labeled with 10 µM bromodeoxyuridine (BrdU) for 20 min. The cells were fixed with 80% methanol on ice for 30 min and permeabilized by treatment with 2 M hydrochloric acid and 0.5% Triton X-100 at room temperature for 45 min. They were then centrifuged, and resuspended in 0.1 M sodium tetraborate solution (pH 8.5) to neutralize the acid. After 30 min incubation with a fluorescein-conjugated anti-BrdU antibody (diluted 1:4; Pharmingen), cells were treated with RNaseA (100 µg/ml) for 15 min at 37°C, and stained with propidium iodide (PI) (10 µg/ml) for 30 min at room temperature. In total, 10,000 cells were analyzed with a flow-cytometer (FACS system, Beckton Dickinson).

**RT-PCR assay**

Total RNA was extracted from cells by the acid guanidinium-phenol-chloroform method. The concentration of RNA was equalized (100 µg/ml). Reverse transcription was carried out using the SUPERSCRIPT Preamplification System (BRL). p53 cDNA was amplified with the following primers: sense, 5'-ATTTGCGTGTGGAGTATTTG-3' and
antisense, 5'-GGAACAAGAAGTGGAGAATG-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified with the primers 5'-ATCATCCCTGCCTCTACTGG-3' (sense) and 5'-CTTCCTCTTGTGCTCTTGCT-3' (antisense). The cDNA (0.1 µg) was added to 99 µl of a PCR mixture containing 200 µM of each dNTP, 0.5 µM of each primer, 1.5 mM MgCl₂, 1xPCR buffer, 7 µM Taq Start Antibody and 2.5 U of Taq Polymerase. PCR was performed for 27 to 36 cycles in an automated thermocycler with the following conditions: denaturing (94°C, 30 sec), annealing (57°C, 1 min) and elongation (72°C, 2 min). To minimize non-specific reactions, the first cycle consisted only of denaturing at 94°C for 3 min. The products (10µl) were run on a 0.8% agarose gel and visualized by ethidium bromide staining.

**Pulse-chase labeling**

Sub-confluent monolayers of Mori cells in 6cm dishes were incubated for 3 days in normal DMEM medium. The cells were incubated for 1.5 h in DMEM containing 10% dialyzed FCS without methionine and cystine, and then labeled with ³⁵S-methionine (ICN) for 2 h in 4.0 ml of medium containing 0.2 mCi³⁵S-methionine. After the labeling, the cultures were rinsed twice with DMEM without methionine and cystine, and then chased for various periods in 4.0 ml of conditioned medium containing 25 mM methionine, 25 mM cystine and 240 mM NaCl. These cells were washed twice with PBS and lysed in RIPA buffer. Radioactive p53 was immunoprecipitated with antibodies against p53 (a mixture of PAb1801 and PAb421) and protein G-Sepharose. After the Sepharose beads had been washed with RIPA buffer, bound proteins were
separated by SDS-PAGE. Gels were fixed and fluorographed in 22.5% PPO (2, 5-diphenyloxazole) in DMSO.

**Microinjection**

Microinjections with glass needles were performed as described elsewhere (45). To identify microinjected cells, cells were plated on glass coverslips on which small circles had been engraved with a diamond knife. Usually 50 to 100 of the cells in each small circle were microinjected for analysis. p38MAPK expression plasmid (10 ng/µl) was microinjected into the nuclei of Mori cells with or without a constitutively active MKK6 (MKK6EE) expression plasmid (5 ng/µl). To see the effect of dominant negative MKK6, MKK6AA expression plasmid (50 ng/µl) was microinjected into Mori cells.

**Luciferase assay**

H1299 cells were transfected with a pRenilla luciferase (RL)-CMV reporter plasmid (0.1 µg) and a luciferase reporter plasmid (0.4 µg) driven by one copy of the p53 responsive element motif of p21WAF1 (46), together with 0.1 µg of the various p53 expression plasmids using Superfect (Qiagen). Cells were harvested 24 h after transfection, and luciferase assays were performed according to the manufacturer’s instructions (Promega). Luciferase activity was normalized to Renilla luciferase activity in each sample. All points are displayed as the mean and standard deviation of triplicate assays.
Results

p53-dependent cell cycle arrest following osmotic shock.

When normal human fibroblasts were cultured continuously in hyperosmotic medium, nuclear accumulation of p53 was detected by immunostaining within hours. The accumulation was induced in medium containing 210–270 mM NaCl, but above 290 mM NaCl, cells shrank in a short time and detached from dishes. In most subsequent experiments, cells were cultured in medium containing 240 mM NaCl. Accumulation of p53 was detected as early as 3 h, and peaked around 6 h, as determined by either immunostaining (Fig. 1, A and B) or Western blotting (Fig. 1C). More than 90% of the treated cells showed positive staining for p53 with a similar level of fluorescence intensity. The amount of p53 protein returned to the basal level within 12 h even in the hyperosmotic medium. p21^{WAF1} is a major target of activated p53 (11), and inhibits the Cdk/Cyclin complex, thus inducing cell cycle arrest at G1/S (47). Following the increase in the p53 protein level, the p21^{WAF1} protein level rose gradually with a peak at around 9 h, and then returned to the basal level within 24 h (Fig. 1D). Accumulation of p53 and p21^{WAF1} with similar time courses was observed in a human mammary carcinoma cell line, MCF7, which has been shown to have normal induction of p21^{WAF1} (48) (data not shown). However, accumulation of p21^{WAF1} was detected in neither p53^{-/-} mouse fibroblasts (41) nor a human lung carcinoma cell line H1299 defective in p53 function (data not shown). These results indicate that the induction of p21^{WAF1} is dependent on p53.

To determine whether up-regulated p21^{WAF1} evoked cell cycle arrest, cell cycle
profiles were analyzed by flow-cytometry. In proliferating normal fibroblasts (Mori), the percentage of cells in the S-phase was 11% (Fig. 2A, C and Table I). However, in hyperosmotic medium, cells in the S-phase disappeared within 12 h (Fig. 2A, 12 h) and cell cycle arrest mainly at the G1-phase continued for more than 24 h (Table I). To confirm that activated p53 is required for cell cycle arrest in high salt medium, we analyzed and compared cell cycle profiles of primary cell strains derived from p53 knock-out and wild-type mice. While p53+/+ fibroblasts showed a marked decrease in the S-phase population and an increase in the G1-phase population 12~24 h after osmotic shock, p53−/− fibroblasts grew without cell cycle arrest (Fig. 2B, 12~24 h and Table I). Since these two types of mouse cells have the same genetic background except for the p53 gene, these results indicate the absolute requirement of p53 for cell cycle arrest at G1/S.

_Transient stabilization of p53 protein through down-regulation of Mdm2 following osmotic shock._

The amount of p53 could be controlled at each step from transcription of the gene through to degradation of the protein. To determine whether transcription levels changed before and after osmotic shock treatment, the total amount of p53 mRNA was measured by an RT-PCR method. As shown in Fig. 3A, no change was observed during at least 6 h incubation. We could not determine the production rates of p53 protein by pulse-labeling with 35S-methionine, because of changes in the incorporation efficiency of the isotope after osmotic shock. To examine changes in the stability of
p53, pulse-chase experiments with $^{35}$S-methionine were performed. As shown in Fig. 3B, while in non-treated cells (Non-treated in Fig. 3B) labeled p53 disappeared rapidly, in osmotic shock-treated cells, the protein was markedly stabilized for at least 4 h. These results suggest that p53 is accumulated mainly because of a transient enhancement of protein stability following osmotic shock.

To analyze the mechanism of stabilization of p53, we determined the Mdm2 protein levels of MCF7 cells following osmotic shock. We chose this cell line since the cells showed accumulation of p53 on osmotic stress and relatively high levels of Mdm2 under normal culture conditions. Mdm2 levels decreased within 1 h following osmotic shock, became minimal at 6 h when the p53 level became maximal, and increased again at 9 h, possibly due to the transcriptional activation by activated p53 (Fig. 3C).

**Phosphorylation of p53 at ser33 and interaction of p53 with p300 following osmotic shock.**

Recent study revealed that the phosphorylation of certain serine residues in the N-terminal region is critical for the stabilization and transcriptional activation of p53 protein (24, 31, 36). These residues include ser20 for stabilization and ser15, 33, and 46 for activation. We determined whether these important serine residues were phosphorylated in normal human fibroblasts following hyperosmotic treatment by using phospho-serine-specific rabbit polyclonal antibodies. While phosphorylation of ser15, 20, and 46 was detected by Western blotting in X-ray irradiated normal human fibroblasts, no positive blotting signals of phosphorylation at these sites were obtained.
in cells treated with osmotic shock during 9 h incubation (Fig. 4A). Even if the cells were cultured in medium containing a higher concentration of NaCl (280 mM) (Fig. 4C) for longer incubation times (up to 12 h), phosphorylation of ser15 and ser46 was not detected (data not shown). In contrast, phosphorylation of ser33 was observed following osmotic and X-ray treatments. Under these conditions, comparable amounts of p53 protein were accumulated following both treatments (Fig. 4A). Phosphorylation of ser15, ser33, and ser46 was also examined by immunostaining. While all of these sites were phosphorylated in X-ray irradiated cells, only ser33 was phosphorylated in osmotic shock-treated cells (Fig. 4B). Because such positive-staining for ser33 was completely blocked with a phospho-peptide used for immunization, this signal was confirmed to be specific (data not shown). Thus, we concluded that among the four phosphorylation sites in the N-terminus of p53, only ser33 was phosphorylated on osmotic treatment in normal human fibroblasts.

The transcriptional coactivator p300 has been shown to interact with p53 following genotoxic treatments and induce acetylation of lys 382 of p53, which is important for activation of p53 (34, 37). As shown in Fig. 4C, both osmotic shock (Osm 240) and X-ray irradiation induced acetylation of lys382 in normal human fibroblasts, while a specific inhibitor of proteasome (lactacystin) failed to induce this modification (Fig. 4C). Osmotic shock with a higher concentration of NaCl (280 mM) also failed to induce the acetylation, possibly due to low levels of p53 accumulation resulting from shrinkage of cells (Fig. 4C). To examine the association of p53 with p300, cell extracts prepared from MCF7 cells were immunoprecipitated with an anti-p300 antibody, and
p53 was detected by Western blotting. An association was detected in cells treated with osmotic shock and X-ray irradiation, but not in cells treated with lactacystin (Fig. 4C).

**p38MAPK is not involved in the stabilization of p53.**

In vivo, it was suggested that p38MAPK phosphorylates ser33 of p53 following genotoxic treatment (31). To examine the effect of p38MAPK on the stability of p53, a specific inhibitor of p38MAPK (SB203580) was included in the hyperosmotic medium at the concentrations of 20 µM~40 µM, and p53 was detected by Western blotting and immunostaining. Comparable amounts of p53 were accumulated both in the presence and in the absence of the inhibitor. Under these conditions, p38MAPK protein levels were also the same (Fig. 5A). On immunostaining, the frequency and intensity of fluorescence of p53-positive cells in response to osmotic shock was similar to those of control cells treated without the inhibitor (data not shown). These results clearly indicate that the p38MAPK inhibitor has no effect on the stabilization of p53 following osmotic shock.

**Requirement of p38MAPK for transcriptional activation of p53.**

To examine the effect of SB203580 on phosphorylation of ser33, human fibroblasts were cultured in hyperosmotic medium containing the p38MAPK inhibitor and stained for phospho-ser33. While nearly 90% of the control cells treated without the inhibitor showed positive staining, only 20% of the cells were positive in the presence of the
inhibitor (Fig. 5, B and C). Similar inhibition of phosphorylation at ser33 was observed with the human lung carcinoma cell line A549 (Fig. 5C). Under these conditions, acetylation of p53 at lys382, induction of p21WAF1, and Mdm2 expression were almost completely inhibited as determined by Western blotting (Fig. 5, D and E). Such inhibition of p21WAF1 induction also occurred with another p38MAPK inhibitor, SB202190 (data not shown).

To examine whether accumulation of p53 with diminished expression of p21WAF1 influenced the cell cycle arrest observed in hyperosmotic medium, cell cycle analysis was carried out with human fibroblasts by flow-cytometry. Whereas the percentage of cells in the S-phase at the 24 h time-point was nearly zero in the absence of the p38MAPK inhibitor, that of cells cultured in hyperosmotic medium in the presence of the inhibitor was 7% (Fig. 5F, SB +). This value was similar to that of cells in normal medium (11%) (Fig. 5F, Cont).

To evaluate the role of phosphorylation of p53 at ser33 in transactivation, a p53 expression plasmid mutated at ser33 (S33A) was co-transfected with a luciferase-reporter plasmid containing the p53-binding site of p21WAF1 into a p53-null cell line (H1299) derived from lung cancer. Although mutated p53 protein was expressed at a level similar to wild type p53 (WT), expression of the reporter gene was inhibited by this substitution (Fig. 5G, S33A). As reported elsewhere (36), substitution of ser15 resulted in a more significant inhibitory effect, and substitution of both ser15 and ser33 acted additively (Fig. 5G, S15, 33A). These results strongly suggest that p38MAPK is the major kinase involved in phosphorylation of p53 at ser33, and that this phosphorylation is
important for cell cycle arrest at G1/S in response to osmotic shock.

**Phosphorylation of p53 at ser33 by p38MAPK in living cells.**

SB203580 is a specific inhibitor of p38MAPK, but we cannot exclude the possibility that it acts simultaneously on some unidentified protein kinase(s) in vivo. To confirm that p38MAPK phosphorylates p53 at ser33 in vivo, primary human fibroblasts were cultured for 2 h in medium containing lactacystine to induce nuclear accumulation of p53. Lactacystine inhibits the action of proteasome (49), thus preventing degradation of p53. p53 accumulated in this way is not phosphorylated at any possible serine residues in the N-terminus (28, 34). When p38MAPK expression plasmid was co-microinjected into the nuclei of the lactacystine-treated cells with a constitutively active MKK6 (MKK6EE) expression plasmid, overexpressed p38MAPK migrated into the nucleus within 3 h (Fig. 6A), and phosphorylated lactacystine-induced endogenous p53 at ser33 (Fig. 6B). However, microinjection of p38MAPK expression plasmid alone resulted in overexpression of p38MAPK in the cytoplasm (Fig. 6A), and no phosphorylation at ser33 was observed (data not shown).

Hemagglutinin (HA) epitope-tagged dominant negative MKK6 (MKK6AA) was localized both in the cytoplasm and the nucleus of human fibroblasts 12 h after microinjection (data not shown). When these cells were further cultured in hyperosmotic medium for 6 h, p53 was accumulated to almost the same level as in non-injected cells, but phosphorylation of ser33 was markedly suppressed (Fig. 6C). In contrast, control cells microinjected with a vector plasmid alone and treated in the
same way, contained a comparable amount of p53 phosphorylated at ser33 (Fig. 6C). These results suggest that p38MAPK can phosphorylate p53 at ser33 in living cells, but that this phosphorylation is not required for stabilization of p53 induced by osmotic shock.

Inhibition of transcriptional activation of p53 in stable transformants expressing dominant negative MKK6.

To support the conclusion made from the microinjection experiments quantitatively, we transfected the HA-tagged dominant negative MKK6 plasmid into human cell lines and obtained several stable transformed clones expressing high levels of HA. Induction of p53 and p21WAF1 accumulation, and phosphorylation of p53 at ser33 were monitored by Western blotting. The A549 cell has been shown to accumulate p53 protein following various types of genotoxic stress (34). When A549 transformants were cultured in hyperosmotic medium, the amount of p53 protein increased to a level similar to that in control cells transformed with an empty vector plasmid (Fig. 7A). However, phosphorylation at ser33 was severely suppressed in the transformants containing dominant negative MKK6 (Fig. 7A). Unfortunately, we could not analyze the effect of dominant negative MKK6 further with A549 transformants, because induction of p21WAF1 was found to be impaired in A549 cells due to an as yet undetermined defect (data not shown). Therefore, we obtained stable transformants of another cell line, MCF7. When MCF7 cells transformed with a dominant negative MKK6 plasmid or an empty vector plasmid were cultured in hyperosmotic medium, similar levels of p53 were
detected in both (Fig. 7B). However, acetylation of p53 at lys382 and induction of 
p21WAF1 were inhibited nearly to the control level in the transformants expressing 
dominant negative MKK6 (Fig. 7, B and C). Because a similar suppression of p53 
phosphorylation at ser33 was observed in two independent stable transformants 
derived from different cell lines (A549 and MCF7), the possibility that the suppression is 
due to unknown effects associated with transformation and selection procedures seems 
unlikely.
Discussion

In this study, we showed that p53 was activated in human cells by hyperosmotic treatment as determined by expression of p21<sup>WAF1</sup>, or cell cycle arrest. This activation was almost completely inhibited by either p38<sup>MAPK</sup>-specific inhibitors (SB203580 or SB202190), or expression of the dominant negative form of MKK6. In mammalian cells, the p38<sup>MAPK</sup> signaling cascade consists of at least three consecutive steps: ASK1, TAK1→MKK3/6→p38<sup>MAPK</sup>. Additional pathways merging at MKK3/6 are also postulated (50). Concomitant with activation of p38<sup>MAPK</sup>, ser33 of p53 was phosphorylated. p38<sup>MAPK</sup>α and β are expressed in normal human fibroblasts and these isoforms are inhibited by SB203580, while p38<sup>MAPK</sup>γ and δ are insensitive to the drug (51). Thus, the results in this study indicate that the MKK6→p38<sup>MAPK</sup> (α or β) pathway is indispensable for osmotic shock-induced p53 activation. Although phosphorylation at ser33 seems essential for the activation of p53, we cannot exclude the possibility that phosphorylation of other sites by activated p38<sup>MAPK</sup> is required for full activation. It was suggested that phosphorylation of ser389 is important in mouse cells for p53-mediated transcriptional activation by UV irradiation (52, 53), and that this phosphorylation is mediated by p38<sup>MAPK</sup> (54, 55). Recently, it was reported that ser33 and ser46 of human p53 are phosphorylated by p38<sup>MAPK</sup> after UV irradiation (31), and that phosphorylation of ser33 by p38<sup>MAPK</sup> is critical for activation of p53 after treatment with DNA damaging agents (32). It is not clear whether this signaling pathway including the sensor is the same as that of osmotic shock, but these results suggest phosphorylation of ser33 to be a general process for activation of p53 following environmental stress. In vitro, p38<sup>MAPK</sup>
can phosphorylate p53 at ser33, but not at ser46 (16, 32). Phosphorylation of ser46 was found to be critical for induction of p53AIP1, a mediator of p53-dependent apoptosis localized in mitochondria (16). Under our experimental conditions of osmotic shock, no cell death was observed. Therefore, our finding that only ser33 is phosphorylated by p38MAPK is consistent with the idea that phosphorylation of ser46 is involved in p53-dependent apoptosis.

Many lines of evidence indicate the significance of phosphorylation at ser15 for activation of p53. This phosphorylation is mediated by either ATM or ATR in X-ray or UV irradiated cells (28, 30). While ser15 is reported to be phosphorylated in murine renal collecting duct cells (mIMCD3) treated with osmotic shock (39), we could not observe any phosphorylation of p53 at this site in normal human fibroblasts under various hyperosmotic conditions. Since mIMCD3 is a murine cell line transformed with SV40, this discrepancy might be due to differences between primary cells and transformed cell lines. Phosphorylation of ser15 stimulates association of p53 with p300/CBP (35, 36). Transcriptional activity of p53 is then enhanced through acetylation of some lysine residues in the C-terminal region. Actinomycin D, however, induces acetylation of p53 in vivo without phosphorylation of ser15 (56), and a p53 N-terminal peptide phosphorylated at ser33 inhibits the acetylation in vitro (34). In the present study, we showed that phosphorylation of ser33 and acetylation of lys382 were almost completely inhibited by either p38MAPK-specific inhibitors or expression of a dominant negative form of MKK6. These results suggest that phosphorylation of only ser33 also stimulates acetylation of p53.
The elevation in intracellular p53 protein levels observed after genotoxic stress is mainly caused by increased stability of p53 in most cases. Following osmotic shock, the degradation rate for p53 also decreased transiently, but ser20 was not phosphorylated in this case. Association of p53 with p38MAPK itself is also reported to induce stability in p53 in vitro (31), but overexpression of p38MAPK alone or with constitutively active MKK6 did not induce accumulation of p53 either in the cytoplasm or in the nucleus (data not shown). In this study, we found that the levels of Mdm2 were down-regulated following osmotic shock. Hypoxia also induces down-regulation of Mdm2 with no change in the Mdm2 transcription rates (26). Recently, it was reported that Mdm2 is conjugated with small ubiquitin–like modifier protein SUMO-1 at lys446 and that this sumoylation inhibits down-regulation of Mdm2 through self-ubiquitination. SUMO-1 modification levels decrease after X-ray and UV irradiation, and this decrease is inversely correlated with the level of p53 (57). Currently, we do not know whether the same mechanism of down-regulation of Mdm2 operates following osmotic shock, but this down-regulation may be at least in part a cause of the transient stabilization of p53 in this case.

Elevated levels of p53 normalized within 12 h, even though cells were cultured continuously in hyperosmotic medium. However, when cells were treated again but with a much higher osmotic shock (300 mM NaCl), p53 accumulation was observed with a time course similar to that of the first treatment (unpublished data). Cells could not survive in the second hyperosmotic medium without “adaptation” to the first treatment (240 mM NaCl). Thus, cells seem to have a sensor to detect differences in osmolarity
between the cytoplasm and the extracellular environment. This signal evokes both the immediate response of p38\textsuperscript{MAPK} (within 5 min) (3) for adaptation to hyperosmotic medium and the late p53 response for regulation of the cell cycle.
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Footnotes

The abbreviations used are: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and rad3 related; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor.
FIG. 1. **Nuclear accumulation of p53 following osmotic shock.** A, Primary human fibroblasts (Mori cells) were cultured in hyperosmotic medium (240 mM NaCl) for the periods indicated. Nuclear accumulation of p53 was detected by indirect immunostaining with a monoclonal antibody against p53 (PAb1801). B, Frequency of p53-positive cells shown in (A) is determined as the percentage of cells showing positive staining after osmotic shock. At least 200 cells were counted for each time point. C, Mori cells were cultured in hyperosmotic medium for various periods. Accumulation of p53 was analyzed by Western blotting with an antibody against p53 (PAb1801). D, Induction of p21WAF1 was determined by Western blotting with an antibody against p21WAF1 (OP64). Whole cell extracts used in (D) were derived from the same samples used in (C).

FIG. 2. **p53-dependent cell cycle arrest at G1/S following osmotic shock.** Cells were double stained for flow-cytometry. DNA was stained with PI and cells in the S-phase were identified by incorporation of BrdU. Distributions of cells at each phase of the cell cycle are shown by density plots. After osmotic shock, cells were cultured for 12 h, 24 h, or 48 h. A, Mori cells. B, Upper panel; p53 +/+ mouse fibroblasts, lower panel; p53 +/- mouse fibroblasts. Control cells before the osmotic shock are indicated by the letter C.

FIG. 3. **Stabilization of p53 protein through down-regulation of Mdm2 following**
osmotic shock. A, No change in p53 mRNA levels before and after osmotic shock. Total RNA was extracted from Mori cells cultured in hyperosmotic medium for the periods indicated. Control samples from normal culture are indicated by the letter C. p53 mRNA levels were determined by an RT-PCR method at different amplification cycles. GAPDH mRNA levels in the same samples used for p53 mRNA were analyzed in the same way as the control of constitutively transcribed mRNA. B, Enhanced p53 stability induced by osmotic shock. Mori cells were labeled for 1.5 h with $^{35}$S-methionine, and then chased with excess cold methionine in hyperosmotic media. At the indicated time points, p53 was immunoprecipitated with a mixture of antibodies against p53 and protein G-Sepharose, and analyzed by autoradiography (Osm). As a control, labeled cells were chased in normal medium (Non-treated). C, Transient stabilization of p53 through down-regulation of Mdm2. MCF7 cells were cultured in hyperosmotic medium (260 mM NaCl) for the periods indicated. Mdm2, p53, and $\alpha$-tubulin were detected by Western blotting. A control sample from normal culture is indicated by the letter C.

FIG. 4. Phosphorylation of p53 at ser33 and interaction of p53 with p300 following osmotic shock. A, No phosphorylation of p53 at ser15, 20, and 46 following osmotic shock. Mori cells were cultured for the periods indicated in hyperosmotic media, or irradiated with X-rays (20 Gy). Phosphorylation of ser15, 20, 33, and 46 was detected by Western blotting using polyclonal antibodies against these p53 phospho-serines. p53 levels were determined in the same way as in Fig. 1C.
Same protein amounts of cell extracts were applied to each lane. A cell extract from normal culture was included in the first lane (Cont). B, Phosphorylation of p53 at ser33 following osmotic shock. Mori cells were cultured for 6 h in hyperosmotic medium (right panels), or cultured for 3 h in normal medium after X-ray irradiation (20 Gy) (middle panels). Non-treated control cells are shown on the left (Cont). Cells were stained for phospho-ser15 (upper), phospho-ser33 (middle), and phospho-ser46 (lower), with rabbit polyclonal antibodies specific for one of the phospho-ser of p53. C, Acetylation of p53 at lys382 following osmotic shock. Upper panel; Normal human fibroblasts (Mori) were cultured for 6 h in hyperosmotic medium (240 or 280 mM NaCl) (Osm 240, Osm 280), cultured for 3 h in normal medium after X-ray irradiation (20 Gy) (IR), or cultured for 3 h in normal medium in the presence of lactacystin (50µM) (Lacta). Phosphorylation of ser15 (phospho-ser15), acetylation of lys382 (Ac382), and accumulation of p53 (p53) were detected by Western blotting. Lower panel; MCF7 cells were cultured for 6 h in hyperosmotic medium (260 mM NaCl) (Osm), cultured for 3 h in normal medium after X-ray irradiation (20 Gy) (IR), or cultured for 3 h in normal medium in the presence of lactacystin (50µM) (Lacta). Cell extracts were immunoprecipitated with an antibody against p300 (N-15) and the co-immunoprecipitated p53 was detected by Western blotting using the DO1 antibody [IP (p300), p53]. Blots were reprobed with an antibody against p300 (N-15) to assess the level of p300 [IP (p300), p300]. p53 protein levels in each cell extract were determined separately by Western blotting (using the DO1 antibody) (Lysate, p53).
FIG. 5. **Inhibition of p53 activation by p38MAPK inhibitors.** A, Mori cells were pre-cultured for 30 min in the presence of 40 µM SB203580 and then cultured for 6 h either in hyperosmotic medium (SB203580 +, Osm +) or in normal medium (SB203580 +, Osm -) containing the inhibitor. As controls, cells were treated in the same way without the inhibitor (SB 203580 -). p53 and p38MAPK were detected by Western blotting using a monoclonal antibody PAb1801, and a polyclonal antibody C-20, respectively. Similar results were obtained in the presence of 20 µM of SB203580. B and C, Inhibition of phosphorylation at ser33 of p53 by SB203580. Mori or A549 cells were cultured for 6 h in hyperosmotic medium in the presence or absence of SB203580 (40 µM). After fixation, these cells were stained for phospho-ser33 of p53 with a rabbit polyclonal antibody. In (B), Mori cells stained for phospho-ser33 are shown on the right with control cells (SB203580 -) on the left. In (C), the effect of SB203580 is quantitated by counting at least 200 cells. D, Inhibition of p53 acetylation by the p38MAPK inhibitor. Mori cells were cultured in hyperosmotic medium in the presence or absence of SB203580 (40 µM) for the periods indicated. Acetylation of lys382 was detected in the same way as in Fig. 4C. E, Inhibition of p21WAF1 and Mdm2 induction by the p38MAPK inhibitor. Mori cells were cultured in hyperosmotic medium in the presence or absence of SB203580 (40 µM). Nine hours later, induction of p21WAF1 and Mdm2 was detected by Western blotting using monoclonal antibodies, OP64 and SMP14, respectively. As controls, cells were cultured in normal medium with or without the inhibitor. p38MAPK was detected as shown in Fig. 5A. F, Effects of SB203580 on cell cycle progression of cells cultured for 24 h in hyperosmotic medium in the presence (SB +), or absence (SB -)
- of SB203580 (40 µM), and then labeled with BrdU for 20 min. After fixation and staining, these cells were analyzed by FACSscan. As a control (Cont), cells cultured in normal medium were analyzed in the same way. G, Effects of amino acid substitution of p53 on expression of the reporter gene. Ser15 (S15A), ser33 (S33A), or both (S15, 33A), were substituted for alanine by site-directed mutagenesis. Each of these p53 expression plasmids or vector alone (pRc-CMV) was transfected into the p53 null-H1299 human lung carcinoma cell line together with a reporter plasmid containing the p53 responsive element of p21WAF1. Twenty four hours after transfection, luciferase activity was determined. To confirm that the transfection efficiency was the same, p53 protein levels were checked by Western blotting as indicated by an arrow.

FIG. 6. Effects of MKK6 on activation of p38MAPK. A, Intracellular localization of p38MAPK with or without activation by MKK6. p38MAPK expression plasmid alone (left) or together with a constitutively active MKK6 (MKK6EE) expression plasmid (right) was microinjected into the nuclei of Mori cells, and cultured for 5.5 h. After consecutive fixation with formalin and methanol, the cells were stained for p38MAPK with a rabbit polyclonal antibody (C-20, Santa Cruz). While inactive p38MAPK was localized mainly in the cytoplasm, activated p38MAPK was imported into the nucleus. B, Phosphorylation of p53 at ser33 by activated p38MAPK. Nuclear accumulation of p53 was induced in Mori cells by treatment with the proteasome inhibitor lactacystine (50 µM) for 2 h. A mixture of p38MAPK and constitutively active MKK6 expression plasmids was then microinjected into these cells and cultured for another 2.5 h. Phosphorylation of p53 at ser33 was
revealed by immunostaining with a rabbit polyclonal antibody against phospho-ser33. Cells indicated by arrows are positive. C, Inhibition of phosphorylation of p53 at ser33 by dominant negative MKK6. Dominant negative MKK6 (MKK6AA) expression plasmid (DN) was microinjected into Mori cells and cultured for 13 h. These cells were cultured for another 6 h in the hyperosmotic medium. Cells were then fixed and double-stained for phospho-ser33 and p53. Control cells injected with an empty plasmid are shown on the left (Cont). Upper (phospho-ser33) and lower (p53) pictures show the same fields.

FIG. 7. Inhibition of p53 activation in stable transformants expressing dominant negative MKK6 following osmotic shock. A, Inhibition of p53 phosphorylation at ser33. Stable transformants of A549 cells expressing dominant negative MKK6 (MKK6AA) (DN) were cultured for 6 h in hyperosmotic medium. Induction of p53 accumulation and phosphorylation of p53 at ser33 were detected by Western blotting before (Cont) or after the osmotic treatment (Osm). Control cells (C) transformed with the empty vector were treated in the same way. B, Inhibition of p21\(^{WAF1}\) induction. Stable transformants of MCF7 cells expressing dominant negative MKK6 (MKK6AA) (DN) were cultured for either 6 h or 9 h in the hyperosmotic medium. Induction of p53 (6 h) or p21\(^{WAF1}\) (9 h) accumulation, was determined by Western blotting before (Cont) or after osmotic shock (6 h, 9 h). C, Inhibition of p53 acetylation. Stable transformants of MCF7 cells expressing dominant negative MKK6 (MKK6AA) (DN) were cultured for either 3 h or 6 h in the hyperosmotic medium. Acetylation of lys382 or accumulation of
p53, was determined by Western blotting before (Cont) or after the osmotic shock treatment (3 h, 6 h). To confirm expression of exogeneous HA-tagged MKK6 and application of similar amounts of cell extracts, Western blotting with an anti-HA antibody (Babco) was included in (A), (B), (C).
| Cells       | G1  | S  | G2/M |
|------------|-----|----|------|
| Mori       |     |    |      |
| C<sup>b</sup> | 62  | 11 | 27   |
| 12 h       | 78  |  4 | 18   |
| 24 h       | 84  |  0 | 16   |
| 48 h       | 80  |  3 | 17   |
| p53 +/-    |     |    |      |
| C<sup>b</sup> | 54  | 26 | 20   |
| 12 h       | 75  |  1 | 24   |
| 24 h       | 75  |  0 | 25   |
| 48 h       | 74  |  3 | 23   |
| p53 +/-    |     |    |      |
| C<sup>b</sup> | 53  | 22 | 25   |
| 12 h       | 53  | 19 | 28   |
| 24 h       | 55  | 17 | 28   |
| 48 h       | 55  | 20 | 25   |

<sup>a</sup> Percentages in each phase were determined from the cell cycle patterns in Fig. 2.

<sup>b</sup> Control cells before the osmotic shock.
FIG. 1.

A

Cont | Osm 3 h | Osm 6 h | Osm 12 h

B

% of p53-Positive Cells

Time (h)

C

Time (h)  C  3  6  9  12  24

p53

D

Time (h)  C  3  6  9  12  24

$^{p21^{\text{WAF1}}}$
FIG. 3.

A

| Cycles | Time (h) |
|--------|----------|
| 27     |          |
| 30     |          |
| 33     |          |

| Cycles | Time (h) |
|--------|----------|
| 30     |          |
| 33     |          |
| 36     |          |

B

| Time (h) |
|----------|
| C        |
| 1        |
| 2        |
| 3        |
| 4        |

Non-treated

Osm

C

| Time (h) |
|----------|
| C        |
| 1        |
| 3        |
| 6        |
| 9        |
| 12       |

Mdm2 →

p53 →

α–tubulin →
FIG. 6.

A  

p38MAPK  

p38MAPK + active MKK6  

B  

C  

phospho-ser33  

Cont  

DN  

p53  

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Hiroto Kishi, Kazumi Nakagawa, Mitsuhiro Matsumoto, Moritaka Suga, Masayuki Ando, Yoichi Taya and Masaru Yamaizumi

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