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Resveratrol-induced Activation of p53 and Apoptosis Is Mediated by Extracellular-Signal-regulated Protein Kinases and p38 Kinase[1]

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ABSTRACT

Resveratrol, a phytoalexin found in grapes, berries, and peanuts, is one of the most promising agents for cancer prevention. Our previous study showed that the antitumor activity of resveratrol occurs through p53-mediated apoptosis. In this study, we have elucidated the potential signaling components underlying resveratrol-induced p53 activation and induction of apoptosis. We found that in a mouse JB6 epidermal cell line, resveratrol activated extracellular-signal-regulated protein kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 kinase and induced serine 15 phosphorylation of p53. Stable expression of a dominant negative mutant of ERK2 or p38 kinase or their respective inhibitor, PD98059 or SB202190, repressed the phosphorylation of p53 at serine 15. In contrast, overexpression of a dominant negative mutant of JNK1 had no effect on the phosphorylation. Most importantly, ERKs and p38 kinase formed a complex with p53 after treatment with resveratrol. Strikingly, resveratrol-activated ERKs and p38 kinase, but not JNKs, phosphorylated p53 at serine 15 *in vitro*. Furthermore, pretreatment of the cells with PD98059 or SB202190 or stable expression of a dominant negative mutant of ERK2 or p38 kinase impaired resveratrol-induced p53-dependent transcriptional activity and apoptosis, whereas constitutively active MEK1 increased the transcriptional activity of p53. These data strongly suggest that both ERKs and p38 kinase mediate resveratrol-induced activation of p53 and apoptosis through phosphorylation of p53 at serine 15.

INTRODUCTION

Resveratrol (3,5,4′-trihydroxystibene), a naturally occurring compound present in grapes and other foods, has been shown to provide cancer chemopreventive effects in different systems based on its striking inhibition of diverse cellular events associated with tumor initiation, promotion, and progression (1, 2). At the molecular level, these effects were related to the inhibition of free radical formation and cyclooxygenase activity, as well as induction of differentiation (1). In addition, resveratrol was shown to be a remarkable inhibitor of ribonucleotide reductase and DNA synthesis with cellular arrest in the S phase or the S-G_2 phase transition (3, 4). However, the precise mechanisms of its antitumorigenic or chemopreventive activities remain largely unknown.

Recently, we and others (5–7) have reported that the cancer chemopreventive activity of resveratrol was related to its ability to trigger apoptosis. We found that in a mouse JB6 epidermal cell line, a well-developed cell culture model for studying tumor promotion (8–12), resveratrol induces apoptosis to inhibit tumor promoter-induced cell transformation through increased transactivation of p53 activity. Regulation of p53 activity is through multiple mechanisms, one of which is phosphorylation (13, 14). Recent studies have shown that phosphorylation of p53 protein at Ser 15 may play a critical role in its stabilization, up-regulation, and functional activation (15–19). Mutation of Ser 15 impaired the apoptotic activity of p53 (17), suggesting a pivotal role for phosphorylation at this site in p53 activation and induction of apoptosis. Therefore, these data pose the intriguing question of whether resveratrol induces p53 phosphorylation at Ser 15 to enhance its transactivation and apoptotic activity. Identifying the kinase(s) that phosphorylates Ser 15 will help to delineate the signaling cascade leading to functional activation of p53 and to better understand the anticancer properties of resveratrol. In mammalian cells, MAP[3] kinases represent a family of Ser/Thr protein kinases comprised of three distinct components: ERKs, JNKs, and p38 kinase. In different cell lines, these kinases have been shown to play an important role in the regulation of apoptosis, cell cycle, and differentiation in response to different stimuli (20–26). Recently, resveratrol was reported to induce activation of ERKs during differentiation of neurons (27). The activation of MAP kinases may occur via their translocation to the nucleus, where they phosphorylate target transcriptional factors such as AP-1 (28–31) and p53 (32–35). Therefore, in the present study, we extended prior observations (5) concerning resveratrol-induced p53 activation and effects on induction of apoptosis. We determined whether resveratrol activates MAP kinases and modulates phosphorylation of p53 at Ser 15 to increase its functional activity. Our data show that in the mouse JB6 epidermal cell line, resveratrol induces p53 phosphorylation at Ser 15 [numbering according to Soussi *et al.* (36)] and activates MAP kinases including ERKs, JNKs, and p38 kinase. We further found that resveratrol-induced apoptosis depends on the activities of ERKs and p38 kinase and their phosphorylation of p53 at Ser 15.

MATERIALS AND METHODS

Plasmids and Reagents. CMV-neo vector plasmid and p53 luciferase reporter plasmid (PG13-Luc) were constructed as reported previously (37, 38). DN mutants of ERK2, p38 kinase, and JNK1 were generous gifts from Dr. Melanie H. Cobb (University of Texas, Dallas, TX; Ref. 39), Dr. Mercedes Rincon (University of Vermont, Burlington, VT; Ref. 40), and Dr. Roger J. Davis (University of Massachusetts, Worcester, MA; Ref. 41), respectively. DA mutants of MEK1 and its vector, pUSEamp(+), were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). PhosphoPlus p44/42 MAP kinase, p38 kinase, and JNK antibody kits; phospho-MKK3/MKK6 antibody, p44/42 MAP kinase, p38 kinase, and JNK assay kits; phospho-specific p53 (Ser 15) antibody, Elk-1, ATF-2, and c-Jun fusion proteins; and phospho-specific Elk-1 (Ser 383), ATF-2 (Thr 71), and c-Jun (Ser 63) antibodies were from New England BioLabs, Inc. (Beverly, MA). Mouse monoclonal IgG against p53 (Ab-1) antibody was from Oncogene Research Products (Cambridge, MA); mouse monoclonal phospho-specific JNK antibody and p53 fusion protein were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); MEK1-specific inhibitor, PD98059, was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA); p38 kinase inhibitor, SB202190, was from Calbiochem (La Jolla, CA); LipofectAMINE and LipofectAMINE PLUS reagents, Eagle's MEM, and DMEM were from Life Technologies, Inc. (Grand Island, NY); FBS was from BioWhittaker, Inc. (Walkersville, MD); and luciferase substrate was from Promega (Madison, WI).

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[3] The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular-signal-regulated protein kinase; JNK, c-Jun NH_2-terminal kinase; MEK1, MAP kinase kinase-1; DNA-PK, DNA-dependent protein kinase; ATM, ataxia telangiectasia-mutated; ATR, ATM-Rad3-related protein; MDM2, murine double minute 2; GST, glutathione *S*-transferase; FBS, fetal bovine serum; CMV, cytomegalovirus; DN, dominant negative; DA, dominant activated.

Cell Culture. JB6 mouse epidermal cell line Cl 41 and its stable transfectants, Cl 41 CMV-neo, Cl 41 DN-ERK2 B_3 $mass_1$, Cl 41 DN-p38 G7, Cl 41 DN-JNK1 $mass_1$, and Cl 41 p53, were cultured in monolayers at 37°C and 5% CO_2 using Eagle's MEM containing 5% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin (30, 37, 42). $p53^{+/+}$ fibroblasts were cultured in DMEM with 10% FBS, 2 mM L-glutamine, and antibiotics (5).

Generation of Stable Cotransfectants. JB6 Cl 41 were transfected with CMV-neo vector with or without the cDNA of DN mutants of ERK2, p38 kinase, and JNK1 by using LipofectAMINE following the manufacturer's instructions. The stable transfectants were obtained by selection for G418 resistance (300 μg/ml) and further confirmed by assay of respective activity as described previously (12, 34, 43).

Immunoblotting and Immunoprecipitation. Immunoblotting for phosphorylated proteins of ERKs, p38 kinase, and JNKs was carried out using phospho-specific MAP kinase antibodies against phosphorylated sites of ERKs, p38 kinase, or JNKs, respectively (12). To study the effect of resveratrol treatment on the induction of p53 phosphorylation at Ser 15 and the interaction of p53 with MDM2, a negative regulatory partner, or ERKs or p38 kinase *in vivo*, p53 protein, ERKs or p38 kinase were first immunoprecipitated with a specific antibody against p53, ERKS, or p38 kinase, respectively. The immunocomplex was then analyzed by SDS-PAGE and immunoblotted with the appropriate antibodies. Briefly, JB6 Cl 41 cells or its transfectants were cultured in 100-mm dishes with 5% FBS MEM until they reached 80–90% confluence. Then, the cells were starved by culturing them in 0.1% FBS MEM for 24 h. After the cells were treated with resveratrol for induction of p53 phosphorylation at Ser 15, the cells were lysed on ice for 30 min in lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium PP_i, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] and centrifuged at 14,000 rpm for 10 min in a microcentrifuge. The lysates containing 500 μg of protein were immunoprecipitated using monoclonal mouse IgG against p53 antibody and protein A/G plus-agarose. The beads were washed extensively to eliminate nonspecific binding, and levels of phosphorylated protein of p53 at Ser 15, p53, and MDM2 proteins and phosphorylated ERKs and p38 kinase were selectively measured by Western immunoblotting using a specific antibody and chemiluminescent detection system.

***In Vitro* Kinase Assays.** Assays of ERKs, p38 kinase, and JNKs were carried out as described in the protocol provided by New England BioLabs, Inc. In brief, JB6 Cl 41 cells or transfectants were starved for 24 h in 0.1% FBS MEM at 37°C, in a 5% CO_2 atmosphere incubator. The cells were treated with resveratrol (20 μM) or its vehicle, DMSO (<0.1%), as negative control for the indicated times. Then, the cells were washed once with ice-cold PBS and lysed in 300 μl of lysis buffer. The lysates were sonicated and centrifuged. Endogenous ERKs, p38 kinase, or JNKs were immunoprecipitated from the supernatant fraction containing 500 μg of protein by incubating with the specific phospho-ERK, p38 kinase, or JNK antibody for 6–10 h at 4°C, followed by incubation with protein A/G plus-agarose for another 4 h. The beads were washed twice with 500 μl of lysis buffer and twice with 500 μl of kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$]. Kinase reactions were performed in 25 μl of the kinase buffer containing the immunoprecipitates and 200 μM ATP at 30°C for 30 min using 2 μg of Elk-1, ATF-2, or c-Jun as substrate for ERKs, p38 kinase, or JNKs, respectively. For p53 phosphorylation, the ERK, p38 kinase, or JNK immunoprecipitates were incubated at 30°C for 60 min in kinase buffer containing 200 μM ATP and 3 μg of p53 as substrate. The phosphorylated proteins were detected by immunoblotting using phospho-specific antibodies.

Assay for p53-dependent Transcriptional Activity. p53-dependent transcriptional activity was assayed by using a Cl 41 cell line stably expressing a luciferase reporter gene controlled by p53 DNA binding sequences (38, 42). Confluent monolayers of Cl 41 p53 cells were trypsinized, and 1×10^4 viable cells, suspended in 100 μl of 5% FBS MEM, were seeded into each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 until the cells reached 80–90% confluence. The cells were starved by culturing them in 0.1% FBS MEM for 24 h. Then the cells were treated with different concentrations of PD98059 or SB202190 for 1 h, followed by treatment with 20 μM resveratrol to induce p53 activity, and cultured for an additional 24 h. The cells were extracted with lysis buffer [100 mM K_2HPO_4 (pH 7.8), 1% Triton X-100, 1 mM DTT, and 2 mM EDTA] and luciferase activity was measured using a luminometer (Monolight 2010). The results were expressed as relative p53 activity (42). To determine whether constitutively active MEK1 induces p53-dependent transcriptional activity, Cl 41 cells or $p53^{+/+}$ fibroblasts were cultured in a 6-well plate until cell density reached 80–90% confluence. Two μg of the p53 luciferase reporter plasmid (PG13-Luc) with 2 μg of plasmid DNA of pUSEamp(+) vector or DA mutants of MEK1 were used to transfect each well by using LipofectAMINE PLUS reagent following the manufacturer's instructions. Twenty-four h posttransfection, the transfectants were subjected to the assay for p53-dependent transcriptional activity as described above.

DNA Fragmentation Assay. Cells were grown in a 10-cm dish, and when cell density reached 80–90% confluence, cells were treated with different concentrations of PD98059 or SB202190 for 1 h followed by treatment with 20 μM resveratrol for 16 h. Both detached and attached cells were harvested by scraping and centrifugation. The cells were then lysed with lysis buffer [5 mM Tris (pH 8.0), 20 mM EDTA, and 0.5% Triton X-100] on ice for 45 min. Fragmented DNA in the supernatant fraction after centrifugation at 14,000 rpm (45 min at 4°C) was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with chloroform and then precipitated with ethanol and 5 M NaCl overnight at −20°C. The DNA pellet was washed once with 70% ethanol and resuspended in Tris-EDTA buffer (pH 8.0) with 100 μg/ml RNase and incubated at 37°C for 2 h. The DNA fragments were separated by 1.8% agarose gel electrophoresis and visualized under UV light (5, 23).

RESULTS

Resveratrol-induced Ser 15 Phosphorylation Is Associated with p53 Stabilization. Previous studies indicated that JB6 Cl 41 cells contain a wild-type p53 protein (38, 42, 44). To identify whether p53 was phosphorylated at Ser 15 *in vivo* in Cl 41 cells treated with resveratrol, a phospho-specific antibody against p53 at Ser 15 (19) was used in Western blot analysis. We found that the level of p53 phosphorylation at Ser 15 was increased by 3-fold 30 min after treatment of cells with resveratrol and reached a maximal induction of 4.5-fold after 2–4 h (Fig. 1*A*). A dose-response study indicated that phosphorylation of p53 at Ser 15 increased in a dose-related way up to 20 μM, but then decreased at 40 μM (data not shown). These results indicated that resveratrol is able to induce p53 phosphorylation at Ser 15. Furthermore, immunoblotting of p53 protein revealed that increased levels of p53 protein correlated well with the observed increase in p53 phosphorylation at Ser 15 (Fig. 1*B*). MDM2 is transcriptionally induced by p53 and works as a feedback inhibitor by promoting p53 protein degradation and inhibiting p53 transcriptional activities (45–47). Here, we also found that MDM2 was induced by resveratrol within 30 min, and the level of MDM2 protein peaked at 2–4 h (Fig. 1*D*), adding further support to the theory that MDM2 is induced in response to p53. However, the amount of MDM2 binding to p53 remained at a constant but low level throughout the time course (Fig. 1*C*). These data are in agreement with previous findings that Ser 15 phosphorylation interferes with MDM2 binding (15, 16) and suggest that resveratrol-induced Ser 15 phosphorylation results in the disassociation of MDM2 and the stabilization of p53.

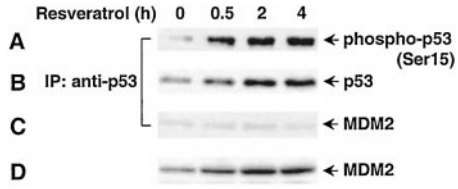

Fig. 1. Resveratrol-induced Ser 15 phosphorylation is associated with p53 stabilization. Serum-starved Cl 41 cells were treated with 20 μM resveratrol for the times indicated. Lysates were prepared from these cells. One-tenth of the Cl 41 lysate volumes was immunodetected with MDM2 antibody (*D*), whereas the rest of them were immunoprecipitated using monoclonal antibodies against p53. The p53 immunoprecipitates were first immunoblotted with a specific antibody against phosphorylation of p53 at Ser 15 (*A*), then stripped and reprobed with antibodies of p53 (*B*) and MDM2 (*C*). *IP*, immunoprecipitate.

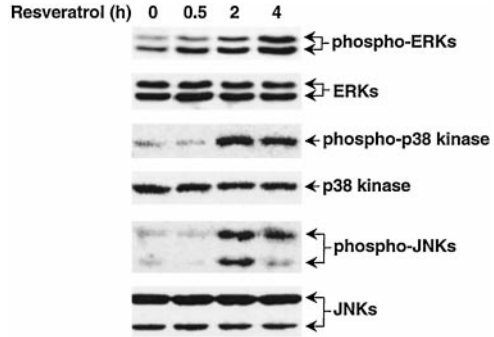

Fig. 2. Resveratrol induces activation of ERKs, p38 kinase, and JNKs. Serum-starved Cl 41 cells were treated with 20 μM resveratrol for the times indicated. The cells were extracted and phosphorylated and total proteins of ERKs and p38 kinase, as well as JNKs, were immunodetected with phospho-specific or total ERKs, p38 kinase, or JNKs antibodies as described by New England BioLabs, Inc. (34, 38, 52).

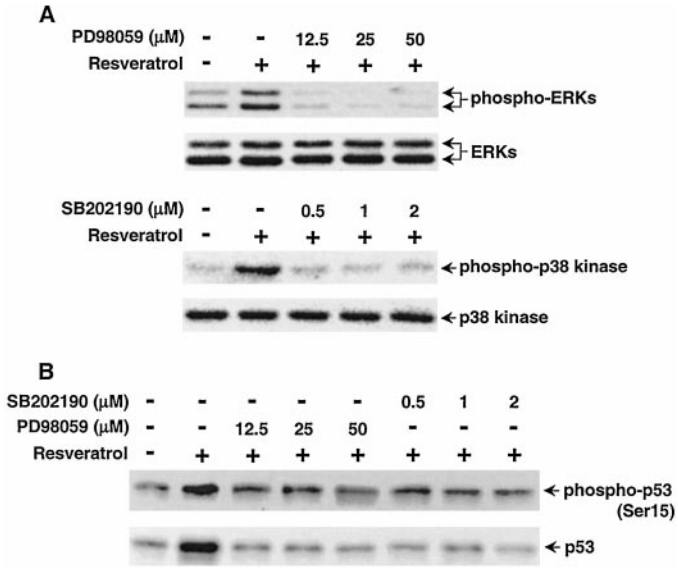

Fig. 3. Inhibitors of MEK1 and p38 kinase abrogate resveratrol-induced p53 phosphorylation at Ser 15. Serum-starved Cl 41 cells were pretreated with MEK1 inhibitor, PD 98059, or p38 kinase inhibitor, SB202190, for 1 h at the concentrations indicated, followed by treatment with 20 μM resveratrol for 2 h (*A*) or 4 h (*B*). Lysates were prepared from these cells, and the phosphorylated and total proteins of ERKs and p38 kinase (*A*), as well as the phosphorylation of p53 at Ser 15 and the level of p53 protein (*B*), were measured as described in the legends to Figs. 1 and 2.

Resveratrol Activates MAP Kinases. Phosphorylation of both Tyr and Thr residues in the activation segment of the kinase domain on MAP kinases is known to be essential for full kinase activity (48, 49). Using phospho-specific antibodies against MAP kinases (12), we found that resveratrol induced a rapid and prolonged activation of ERKs (0.5–4 h), whereas the activation of p38 kinase and JNKs appeared at 2–4 h (Fig. 2). These data suggest a possible role of MAP kinases acting upstream of p53 in a signal transduction pathway initiated by resveratrol.

Inactivated ERKs and p38 Kinase, but not Inactivated JNKs, Block Resveratrol-induced p53 Phosphorylation at Ser 15. To determine whether activation of MAP kinases is involved in resveratrol-induced p53 phosphorylation at Ser 15 *in vivo*, we used two approaches to inactivate ERKs, p38 kinase, or JNKs. First, PD98059, a specific inhibitor of MEK1 that acts by inhibiting activation of ERKs (50) and SB202190, a specific inhibitor of p38 kinase (51), were tested for their effect on resveratrol-induced Ser 15 phosphorylation of p53. Pretreatment with 12.5–50 μM PD98059 or 0.5–2 μM SB202190 markedly inhibited resveratrol-induced activation of ERKs or p38 kinase (Fig. 3*A*) and reduced the phosphorylation of p53 at Ser 15 and the levels of p53 protein (Fig. 3*B*). These data suggest that the intrinsic kinase activities of ERKs and p38 are required for resveratrol-induced p53 phosphorylation at Ser 15 and the stabilization of p53. The second strategy used to inactivate ERKs, p38 kinase, or JNKs was to use DN mutants of these MAP kinases. The Cl 41 cells stably expressing the genes were reported previously (12, 31, 34, 47) and described in "Materials and Methods." Overexpression of DN-ERK2, DN-p38 kinase, or DN-JNK1 specifically blocked resveratrol-induced phosphorylation of ERKs, p38 kinase, or JNKs, respectively, and their respective activity (Fig. 4*A*). Overexpression of DN-ERK2 has been shown to have no effect on activation of JNKs and p38 kinase in DN-ERK2 tranfectants; overexpression of DN-p38 kinase had no effect on activation of ERKs and JNKs in DN-p38 kinase transfectants; and overexpression of DN-JNK1 had no effect on activation of ERKs and p38 kinase in DN-JNK1 transfectants (12, 31, 34, 52). The expression of DN-ERK2 or DN-p38 kinase markedly inhibited p53 phosphorylation at Ser 15 after exposure of cells to resveratrol for up to 4 h (Fig. 4*B*). In contrast, overexpression of DN JNK1 did not result in significant inhibition of p53 phosphorylation at Ser 15 (Fig. 4*B*). These two experiments provide strong evidence that

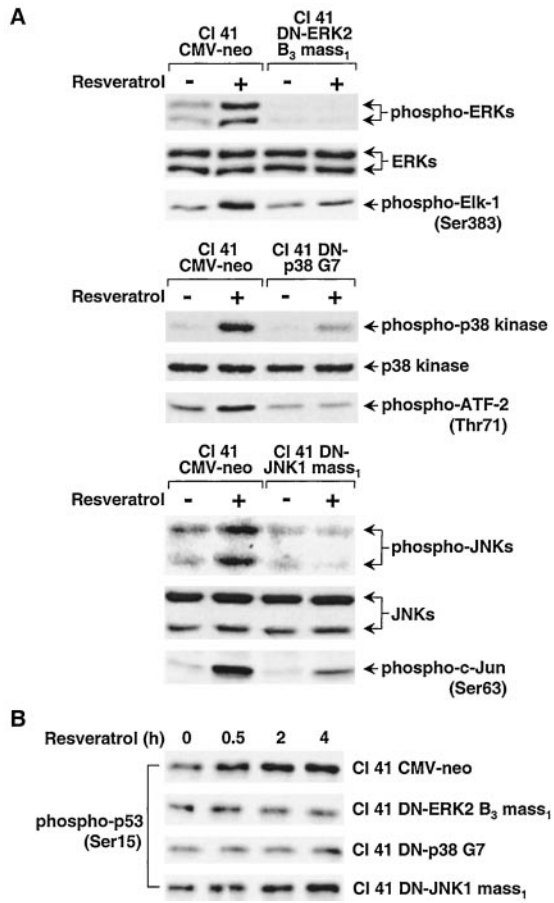

Fig. 4. Expression of DN-ERK2 or -p38 kinase, but not -JNK1, blocks Ser 15 phosphorylation of p53 induced by resveratrol. In *A*, serum-starved Cl 41 cell stable transfectants, Cl 41 CMV-neo and Cl 41 DN-ERK2 B_3 mass$_1$, were treated with 20 μM resveratrol for 4 h, or Cl 41 CMV-neo, Cl 41 DN-p38 G7, and Cl 41 DN-JNK1 mass$_1$ were treated with 20 μM resveratrol for 2 h to induce their respective activity. Lysates were prepared from these cells and the phosphorylated and total proteins of ERKs, p38 kinase or JNKs, as well as the activities of ERKs, p38 kinase or JNKs, were determined as described in "Materials and Methods." In *B*, serum-starved Cl 41 cell stable transfectants as indicated were treated with 20 μM resveratrol for the times designated. Lysates were prepared from these cells, and the phosphorylation of p53 at Ser 15 was measured as described in the legend to Fig. 1.

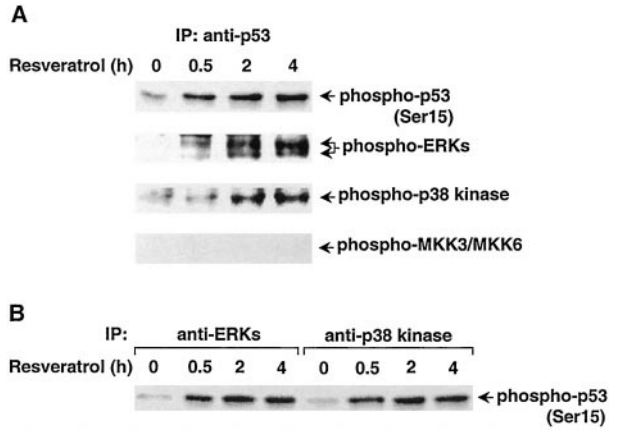

Fig. 5. ERKs and p38 kinase associate with p53 during its phosphorylation at Ser 15 induced by resveratrol. Serum-starved Cl 41 cells were treated with 20 μM resveratrol for the times designated. Lysates were prepared from these cells and immunoprecipitated using monoclonal antibodies against p53 (*A*), ERK or p38 kinase (*B*). The p53 immunoprecipitates were first immunoblotted with a specific antibody against phosphorylation of p53 at Ser 15, then were stripped and reprobed with phospho-ERKs, p38 kinase, or MKK3/MKK6 antibodies. The ERKs or p38 kinase immunoprecipitates were immunodetected with a specific antibody against phosphorylation of p53 at Ser 15.

in Cl 41 cells, resveratrol-induced phosphorylation of p53 at Ser 15 is mediated through ERKs and p38 kinase, but not JNKs.

ERKs and p38 Kinase Are Associated with p53 Phosphorylation at Ser 15 in Resveratrol-treated Cl 41 Cells. Because the above data revealed the important role of ERKs and p38 kinase in the signaling pathway leading to phosphorylation of p53 at Ser 15, we explored whether a direct interaction might exist between p53 and ERKs or p38 kinase. We exposed Cl 41 cells to resveratrol for different periods of time and incubated the cell lysates with specific monoclonal antibodies against p53, ERKs, or p38 kinase and protein A/G plus-agarose as described in "Materials and Methods." Results indicated that phosphorylated ERKs or p38 kinase could be detected in the p53 immunoprecipitated complex, whereas no phosphorylation of MKK3/MKK6, upstream kinases of p38 kinase, was observed (Fig. 5*A*). The kinetics of p53 phosphorylation at Ser 15 correlated well with that of ERKs or p38 kinase phosphorylation induced by resveratrol. Conversely, Ser 15 phosphorylation of p53 was detected in the ERKs or p38 kinase immunoprecipitates from resveratrol-treated Cl 41 cells (Fig. 5*B*). These data show that resveratrol induces the formation of a complex between p53 and ERKs or p38 kinase, which suggests that activated ERKs or p38 kinase may be responsible for p53 phosphorylation at Ser 15.

p53 Is Phosphorylated at Ser 15 *in Vitro* by Resveratrol-activated ERKs and p38 Kinase, but not by Activated JNKs. To test whether ERKs and p38 kinase phosphorylated p53 at Ser 15 directly, we performed immune complex kinase assays of resveratrol-activated ERKs, p38 kinase and JNKs using a full-length GST-p53 fusion protein as the exogenous substrate. This experiment revealed that the exogenous p53 protein was phosphorylated at Ser 15 by early (30 min) and late (4 h) resveratrol-activated ERKs or p38 kinase, but not by activated JNKs (Fig. 6, *upper band*). Activated JNKs, however, did exert activity in phosphorylating c-Jun (Fig. 4*A*). Intriguingly, resveratrol-induced endogenous phosphorylated p53 at Ser 15 mediated by ERKs and p38 kinase was found in the anti-phospho-ERKs, p38 kinase, and JNKs immunoprecipitates (Fig. 6, *lower band*). These results, taken together with the other results from this study, strongly demonstrate that ERKs and p38 kinase are direct mediators of resveratrol-induced p53 phosphorylation at Ser 15.

Inhibition of ERKs and p38 Kinase Reduces Resveratrol-induced p53-dependent Transcriptional Activity and Apoptosis, whereas Activated MEK1 Increases the Transcriptional Activity of p53. To assess the functional consequences of ERK and p38 kinase mediation of phosphorylation of p53 at Ser 15, we first determined the

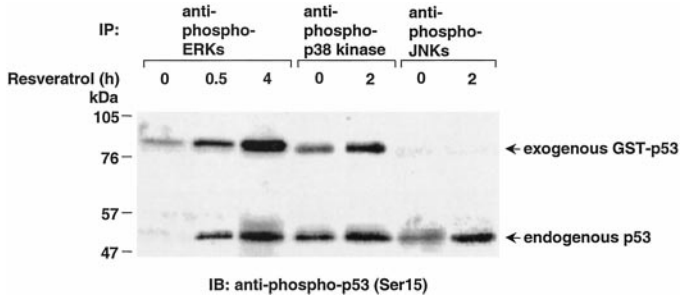

Fig. 6. p53 is phosphorylated at Ser 15 *in vitro* by resveratrol-activated ERKs and p38 kinase, but not by JNKs. Serum-starved Cl 41 cells were treated with 20 μM resveratrol for 30 min or 4 h in ERK assay or for 2 h in the assays of p38 kinase and JNK. Lysates were prepared from these cells, and the immunoprecipitated phospho-ERKs, p38 kinase, or JNKs were assayed for kinase activity by adding purified GST-p53 as exogenous substrate. Ser 15 phosphorylation of exogenous and endogenous p53 was detected as described in the legend to Fig. 1. *IB*, immunoblotting. *kDa*, M_r in thousands.

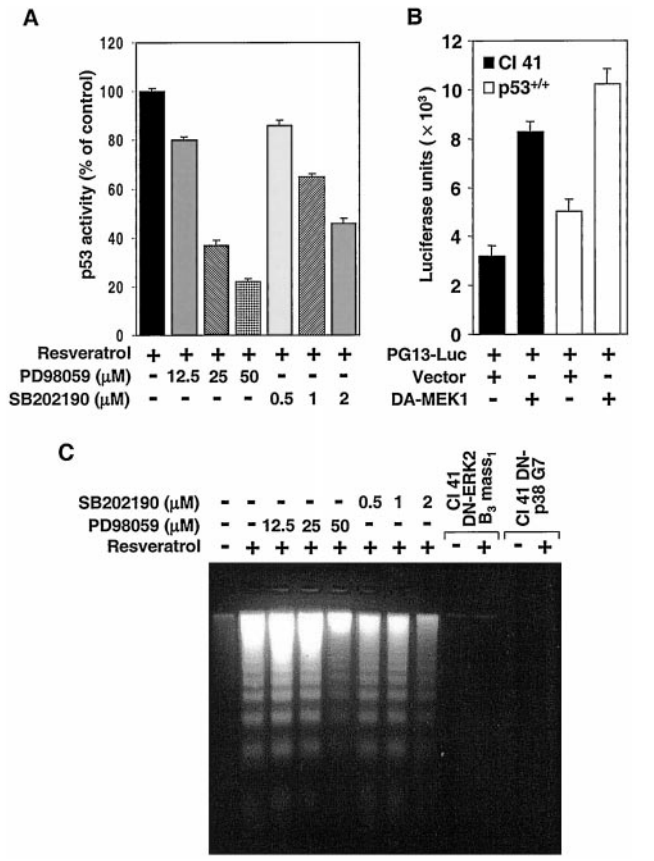

Fig. 7. Inhibition of ERKs and p38 kinase impairs the resveratrol-induced p53-dependent transcriptional activity and apoptosis, whereas activated MEK1 increases the transcriptional activity of p53. In *A*, serum-starved Cl 41 p53 transfectants were pretreated with PD98059 or SB202190 for 1 h at the concentrations indicated. The cells were subsequently treated with 20 μM resveratrol for 24 h. Luciferase activity was expressed as relative p53 activity (% of control). Data from three independent experiments were averaged and are presented as mean ± SE. In *B*, Cl 41 cells or $p53^{+/+}$ fibroblasts were cotransfected with pUSEamp(+) vector or DA-MEK1 and a PG13-Luc reporter plasmid. Twenty-four h posttransfection, the transfectants were assayed for p53-dependent transcriptional activity as described in *A*. *C*, Cl 41 cells were pretreated with various concentrations of PD98059 or SB202190 for 1 h. Then the Cl 41 cells or Cl 41 cell stable transfectants as indicated were treated with 20 μM resveratrol for 16 h and assessed for DNA fragmentation assay as described in "Materials and Methods."

inhibitory effects of PD98059 or SB202190 on resveratrol-induced p53-dependent transcriptional activity. Our results showed that resveratrol-induced p53-dependent transcriptional activity was impaired by pretreatment of cells with PD98059 or SB202190 in the same dose range that inhibited the phosphorylation of p53 at Ser 15 (Fig. 7*A*). Thus, decreased phosphorylation of p53 at Ser 15 was accompanied by a decrease in p53-dependent transcriptional activity. Furthermore, expression of DA mutant of MEK1 (DA-MEK1) in Cl 41 cells or $p53^{+/+}$ fibroblasts showed a marked increase in p53-dependent transcriptional activity (Fig. 7*B*). Our previous study showed that resveratrol induces apoptosis through a p53-dependent pathway (5). To analyze the role of ERKs and p38 kinase in the regulation of p53-mediated apoptosis, we examined apoptosis by assessing DNA fragmentation after inhibitor treatment or by using the DN mutant of ERK2 or p38 kinase. We found that both PD98059 and SB202190 significantly inhibited resveratrol-induced apoptosis (Fig. 7*C*). Additionally, overexpression of DN-ERK2 or DN-p38 kinase also blocked the induction of apoptosis (Fig. 7*C*). These data indicate that through their phosphorylation of p53 at Ser 15, ERKs and p38 kinase play a significant role in resveratrol-induced p53-dependent transcriptional activity as well as p53-mediated induction of apoptosis.

DISCUSSION

Chemoprevention, which refers to the use of nontoxic chemical substances to inhibit, delay, and/or reverse cellular events associated with carcinogenesis, is regarded as a promising alternative strategy to therapy for the management of cancer (53). A vast variety of naturally occurring substances have been shown to protect against experimental carcinogenesis and an increasing amount of evidence suggests that certain phytochemicals, particularly those included in our daily diet, have marked cancer chemopreventive properties (54, 55). Resveratrol is one such dietary chemopreventive phytochemical that has recently attracted considerable interest because of its remarkable multifunctional inhibitory effects on multistage carcinogenesis (1). Our previous data indicated that one of the plausible mechanisms that could account for the chemopreventive activity of resveratrol occurs through p53-mediated apoptosis. To further understand the molecular mechanistic basis for the chemopreventive properties of resveratrol, we demonstrated that resveratrol induced Ser 15 phosphorylation of p53 and activated MAP kinases including ERKs, JNKs, and p38 kinase in Cl 41 cells. Inhibition of ERKs or p38 kinase using PD98059 or SB202190, a specific inhibitor of MEK1 or p38 kinase, respectively, or expression of a DN mutant of ERK2 or p38 kinase impaired the phosphorylation of p53 at Ser 15. Interestingly, we found that phosphorylated ERKs and p38 kinase were present in p53 immunoprecipitates, whereas Ser 15 phosphorylation of p53 was detected in the ERKs or p38 kinase immunoprecipitates from resveratrol-treated Cl 41 cells. Most importantly, resveratrol-activated ERKs or p38 kinase was shown to phosphorylate the p53 protein at Ser 15 *in vitro*. These findings are biologically relevant because resveratrol-induced p53 transcriptional activity and p53-dependent apoptosis were blocked by inhibiting ERKs or p38 kinase activity, whereas constitutively active MEK1 increased the p53-dependent transcriptional activity. All of these data clearly demonstrate that ERKs and p38 kinase play a critical role in resveratrol-induced phosphorylation of p53 at Ser 15, which suggests that induction of both p53 transcriptional activity and apoptosis by resveratrol depends on the activities of ERKs and p38 kinase and their phosphorylation of p53 at Ser 15.

Phosphorylation of p53 at Ser 15 has been reported to be induced in response to a variety of DNA-damaging agents (15, 16). Phosphorylation of Ser 15, a key target during p53 activation, is critical for p53-dependent transactivation (16, 19). Biochemical data indicate that stimulation of p53-dependent transactivation by Ser 15 phosphorylation occurs through decreased binding of p53 to its negative regulator MDM2 (15) and increased binding of p53 to the p300 coactivator protein (18). Substitution of Ala for Ser 15 inhibits p53 apoptotic activity (17) and reduces the ability of p53 to inhibit cell cycle progression (56). Three protein kinases have been reported to phosphorylate p53 at Ser 15 *in vitro*: DNA-PK (15), ATM (57), and ATR (58), all of which are members of the phosphoinositide 3-kinase family. Cells lacking DNA-PK or ATM, however, are still capable of phosphorylating p53 at Ser 15 after DNA damage (16, 57, 59). ATR was recently shown to phosphorylate p53 at Ser 15 *in vitro*, but the level of intrinsic ATR is still low during p53 activation (58). On the other hand, ATR does not mediate Ser 15 phosphorylation induced by the topoisomerase I inhibitor, CPT (58). These results suggest either that these kinases do not play a direct role in Ser 15 phosphorylation *in vivo* or that other multiple kinases are involved in signaling to induce the phosphorylation. ERKs have been shown to phosphorylate p53 at Thr residues 73 and 83 (32), which lie outside the NH_2-terminal transactivation domain (amino acids 1–42) of p53, and Ser 34 is a target for phosphorylation by JNK (33). Very recently, we reported that p38 kinase mediates UV-induced p53 phosphorylation at Ser 389 (34), and another group demonstrated that p38 kinase phosphorylates human p53 at Ser 33 (Ser 34 of mouse p53) and Ser 46 *in vitro* (35). However, the p53 phosphorylation sites mediated by MAP kinases are not completely identified, and less is known about the physiological role of MAP kinases in activating p53-mediated gene transcription and apoptosis. In addition to DNA damage responses, we also noted that the phosphorylation of p53 at Ser 15 could be induced by a phorbol ester or by growth factors such as epidermal growth factor, which both are strong activators of ERKs (30, 43), whereas overexpression of DN-ERK2 could substantially inhibit the phosphorylation (data not shown). Thus, our results indicate that ERK and p38 kinase are primarily responsible for Ser 15 phosphorylation induced by resveratrol.

The JNKs and p38 kinase pathways are associated with increased apoptosis, whereas the ERKs pathway is shown to suppress apoptosis (20). In our study, however, inhibiting ERKs reduced resveratrol-induced apoptosis in a p53-dependent manner. This difference may result from species or cell type differences or different extracellular stimuli. This explanation was supported by several recent findings that the ERKs pathway can trigger cellular apoptosis and predict chemosensitivity of the tumors (21, 23, 25, 60, 61). We also explored the possible involvement of other MAP kinases such as JNKs, which were shown not to be involved in phosphorylation of Ser 15 of p53 (Fig. 4*B* and Fig. 6, *upper band*), although phosphorylated p53 at Ser 15 associates with activated JNKs (Fig. 6, *lower band*). The role of JNK appears to be more than phosphorylation, because it was recently reported to bind to and degrade p53 in a MDM2-independent fashion when this kinase was in an inactive (dephosphorylated) form (62). On the other hand, on activation, JNK stabilizes and activates p53, probably by phosphorylating it at site(s) other than Ser 15 (63). The evidence from this study using JB6 cells demonstrates that activation of both ERKs and p38 kinase by resveratrol is required for Ser 15 phosphorylation of p53 and its activation. ERKs activity precedes p38 kinase activation induced by resveratrol (Fig. 2) and is responsible for early- and late-phase phosphorylation of p53 at Ser 15, whereas p38 kinase may cooperate with ERKs to phosphorylate Ser 15 of p53 in the late phase (Fig. 6). Although the significance for both ERKs and p38 kinase being required for p53 activation and p53-mediated apoptosis is not presently known, some evidence indicates that cross-talk between ERKs and p38 kinase signaling may play an important role in determining cell survival or death (20, 64).

Overall, we demonstrate that ERKs and p38 kinase can physically

interact with and phosphorylate p53 at Ser 15 in response to resveratrol, both *in vivo* and *in vitro*. We propose a model that resveratrol-activated ERKs and p38 kinase bind to p53 molecules to form a complex, leading to phosphorylation of p53 at Ser 15 or other phosphorylation sites, thereby enhancing its functional activities. Our data are in agreement with the recent proposal that to achieve optimal activity, p53 NH_2-terminal sites may be phosphorylated by a p53-associated complex containing several kinases (65). Collectively, our data identify ERKs and p38 kinase as direct signal mediators of resveratrol-induced p53 phosphorylation at Ser 15, and both ERKs and p38 kinase are functionally required for p53-dependent transcriptional activity and apoptosis induced by resveratrol. These data provide a mechanistic basis for the chemopreventive properties of resveratrol.

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