Inhibition of Protein Phosphatase Activity Induces p53-dependent Apoptosis in the Absence of p53 Transactivation*

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Inhibitors of type 1 and type 2A protein phosphatases were used to examine the involvement of protein phosphorylation in regulating the functions of endogenous p53. Exposure of Balb/c 3T3 cells to okadaic acid, an inhibitor of protein phosphatases 1 and 2A, increased the phosphorylation of p53 without changing p53 levels. Okadaic acid treatment enhanced the binding of p53 to a consensus DNA target sequence and caused a 5–8-fold increase in p53 transcriptional activity. Transient expression of SV40 small tumor antigen, a specific inhibitor of protein phosphatase 2A, caused a 4-fold increase in p53 transcriptional activity. Incubation of Balb/c 3T3 cells with okadaic acid also induced programmed cell death in a dose- and time-dependent manner. Decreases in viability, morphological changes, and the appearance of DNA fragmentation were dependent on p53 since cells lacking functional p53 were resistant to okadaic acid-induced apoptosis. The p53-dependent apoptosis induced by okadaic acid was rapid and did not require p53 transcriptional activity. The fact that SV40 small tumor antigen did not induce apoptosis provides additional evidence that p53 transcriptional activity is not sufficient for p53-mediated apoptosis. These results indicate that signaling pathways involving protein phosphorylation play critical roles in controlling the apoptotic activity of p53. Furthermore, a basal level of protein phosphatase 1 or 2A activity is necessary to prevent p53-dependent apoptosis.

The tumor suppressor p53 is a nuclear phosphoprotein that plays a pivotal role in suppressing cellular transformation and tumorigenesis. The p53 gene is frequently lost or rearranged in a large variety of human cancers (1). Biological functions of p53 include induction of cell cycle arrest or programmed cell death in response to DNA damage (2, 3). p53 forms a tetramer that binds specific DNA sequences (4, 5) and activates transcription (6–8). Transcriptional targets of p53 include p21 (9, 10), mdm2 (11, 12), bax (13), gadd45 (14), IGF-BP3 (15), and cyclin G (16, 17). Many of these genes are involved in regulating the cell cycle or apoptosis, suggesting that at least some actions of p53 are mediated by its transcriptional activity. However, transcriptional activation is not always sufficient since, under some conditions, p53 can induce programmed cell death in the absence of transcription (18–20).

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Post-translational modification by phosphorylation is thought to be an important mechanism that regulates p53 function. p53 is phosphorylated at multiple sites in vitro and in vivo. Protein kinases implicated in the phosphorylation of p53 include casein kinase 1 (21), casein kinase 2 (22, 23), cyclin-dependent kinases (24–28), DNA-activated kinase (29), mitogen-activated protein kinase (30), e-Jun N-terminal kinase (31), protein kinase C (32, 33), SV40 large T antigen-activated kinase (34, 35), and Raf-1 (36). The casein kinase 2 phosphorylation site within the C-terminal oligomerization domain (serine 386 in mouse p53) is important in tumor suppression (37). Phosphorylation of the cyclin B/p34cdc2 complex (serine 309 in mouse p53) stimulates DNA binding activity (38). Phosphorylation of the protein kinase C site (serine 370 in mouse p53) also stimulates DNA binding activity in vitro (39). Three phosphorylated serine residues in the N-terminal transactivation domain (serines 6, 15, and 34 in mouse p53) are important for transcriptional activity (39). Except for the protein kinase C site, all of these sites have been shown to be phosphorylated in vivo (40).

p53 is also dephosphorylated by multiple protein-serine/threonine phosphatases. In vitro, p53 can be dephosphorylated by both PP1 (1) and PP2A (33, 41). Dephosphorylation of p53 by PP2A in vitro is inhibited by SV40 small t antigen, a specific inhibitor of PP2A activity (41). Treatment of cells with PP1- and PP2A-specific inhibitors, including okadaic acid, results in the accumulation of hyperphosphorylated p53 (42–44). Okadaic acid-induced phosphorylation of ectopically expressed p53 has been correlated with increases in DNA binding activity and decreased transcriptional activity (43, 44). Okadaic acid also induces programmed cell death in many cell types (45–47), suggesting that PP1 and PP2A are components of signaling pathways that regulate apoptosis. Since p53 is an inducer of apoptosis, the effects of phosphatase inhibitors suggest a potential link between increased p53 phosphorylation and apoptosis. However, an involvement of p53 in the apoptotic response to phosphatase inhibitors has not been demonstrated.

Neither the effects of okadaic acid on endogenous p53 activity nor the relationship between okadaic acid-induced apoptosis and p53 phosphorylation has been examined. We used okadaic acid and SV40 small t antigen to determine the associations among p53 phosphorylation, transcriptional activity, and apoptosis in cells expressing endogenous levels of p53. Okadaic acid-induced hyperphosphorylation of p53 correlated with increased DNA binding activity. Both okadaic acid and small t antigen caused an increase in p53 transcriptional activity. The data also show that okadaic acid induces p53-dependent apoptosis that is not dependent on p53 transcriptional activity.

¶ The abbreviations used are: PP1 and PP2A, protein-serine/threonine phosphatases 1 and 2A, respectively; DMEM, Dulbecco’s modified Eagle’s medium.
Cell Lines, Plasmids, and Transfections—Balb/c 3T3 cells are an immortal line, derived from Balb/c mouse embryo fibroblasts, and express wild-type p53. Mouse (103 p53−/− cells (p53−/−) are a derivative of Balb/c 3T3 that produce a p53 transcript with a stop codon at amino acid 173 and do not express detectable amounts of p53 (48). The (103 175.1 cell line (175.1) was derived from p53−/− after stable transfection with the murine p53 mutant containing a histidine to asparagine substitution at codon 175 (49). The Balb/c 3T3 cell lines were kindly provided by Dr. Arnold J. Levine (Princeton University). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum in an atmosphere containing 5% CO2.

The pGFP.PA.8 reporter plasmid has a basal promoter containing the SV40 early promoter region coupled to the coding sequence for firefly luciferase.5 The p53CON-Luc reporter plasmid was generated by inserting an oligonucleotide containing a p53 consensus binding site upstream of the basal promoter of pGFP.PA.8 (5). The pCMV-lucZ plasmid contains the bacterial β-galactosidase gene driven by the early promoter of human cytomegalovirus, pCMV-SV40-t and pCMV-SV40-Δt are plasmids that express full-length SV40 small t antigen or a truncated small tumor antigen lacking the C terminus (amino acids 111–174). The SV40-Δt protein is incapable of interacting with PP2A (50). pCMVs is the empty vector that was used for the construction of pCMV-SV40-t and pCMV-SV40-Δt.

Log-phase cells (1.105 cells/100-mm dish) were cotransfected with 8 μg of p53CON-Luc or pGFP.PA.8 and 4 μg of pCMV-lucZ mixed with 50 μl of LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Following transfection, cells were incubated for 18 h prior to the addition of fresh medium containing okadaic acid (LC Sciences Corp., Woburn, MA) dissolved in MeSO. Control incubations included the same amounts of MeSO (final concentration of 0.05%) lacking okadaic acid. After treatment, cells were collected, washed with ice-cold phosphate-buffered saline, and solubilized using the lysis reagent provided with the luciferase assay system (Promega, Madison, WI). Luciferase and β-galactosidase (51) activities were measured 48 h following transfections. Luciferase activity was normalized for transfection efficiency using the activity of β-galactosidase.

For experiments with SV40 small tumor antigen, transfections were performed as described above except that 8 μg of pCMV-SV40-t, pCMV-SV40-Δt, or the empty pCMV vector were used in combination with 2 μg of either p53CON-Luc or pGFP.PA.8.

Cell Viability Assay—Cells were seeded in 24-well plates at 1.104 cells/well and grown for 16 h. Okadaic acid was added, and cells were incubated for the times indicated on the figures. Cell viability was determined using trypan blue exclusion. When cycloheximide and actinomycin D were used, they were added 1 h before the addition of okadaic acid.

Northern Blot Analysis—Log-phase cells were treated with combinations of okadaic acid and actinomycin D, and poly(A)+ RNA was isolated by oligo(dT) affinity chromatography using the mRNA isolation system (Invitrogen, San Diego, CA). Three μg of poly(A)+ RNA were separated by electrophoresis on a 7% formaldehyde, 1.0% agarose gel and transferred to a nylon membrane. The membranes were hybridized with a 32P-labeled p21 probe. The membrane was stripped and rehybridized with a 32P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA fragment to confirm equal loading of the RNA samples.

DNA Fragmentation Assay—Cells were treated with 500 nM okadac acid for the times indicated, and low molecular weight DNA was prepared using a modification of a method described previously (52). Cells were lysed at 1.105 cells/ml of cell lysis buffer (10 mM NaCl, 1 mM EDTA, 1% SDS, 10 mM Tris-HCl, pH 7.8). The lysates were treated overnight at 37 °C with 0.5 mg/ml protease K. The next day, NaCl was added to a final concentration of 1.5 M, and the samples were centrifuged at 13,000 × g for 15 min. The supernatants were transferred to a new tube, and an equal volume of ethanol was added to precipitate the DNA. DNA was dissolved in 1% SDS and ethanol at 80 °C prior to analysis. Cell lysates were prepared by bubbling the thawed cells at 4 °C for 1 h and 3 ml of a buffer containing 1% Triton X-100, 0.5% sodium deoxycholic acid, 150 mM sodium chloride, 5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonlfyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 50 μM Tris-HCl, pH 8.0. Insoluble debris was removed by centrifugation at 3000 × g for 15 min at 4 °C. The supernatants were collected and precleared by incubation with 5 μg of normal mouse IgG (Sigma) and 30 μl of protein A-agarose for 1 h at 4 °C followed by centrifugation. One-third of the precleared lysates, containing equal amounts of trichloroacetic acid-precipitable sulfur, were incubated for 2 h at 4 °C with 1 μg of Pab421 or 1 μg of control Pab419 antibody. Twenty μl of protein A-agarose were added, and the mixtures were incubated for 16 h at 4 °C with rocking. The agarose beads were washed three times with centrifugation and washed five times with 1 ml of ice-cold cell lysis buffer. The material on the beads was released by centrifugation at 10,000 × g for 10 min at 4 °C. DNA was precipitated using a modification of a protocol previously described (58). Cells (1.105) were treated with 500 nM okadac acid. Cells were collected at 4 °C by centrifugation for 5 s in an Eppendorf microcentrifuge set at maximum speed. The pellet, containing crude nuclei, was resuspended in 200 μl of ice-cold buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM sodium pyrophosphate, 1 mM sodium orthovandate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin), and KCl was added to a final concentration of 500 mM. The nuclei were extracted at 4 °C for 60 min with gentle shaking. An equal volume of ice-cold buffer C (20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.2 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM

2 R. S. Williams, personal communication.

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phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin) was added, and the mixture was centrifuged for 10 min at 4 °C in an Eppendorf microcentrifuge set at maximum speed. The supernatant was collected, aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C. Protein was quantitated using the modified BCA assay described above.

Prior to assay, each nuclear extract was diluted with buffer C to a final KCl concentration of 100 mM. Binding reactions included 1 ng of 

\[ ^{32}P \]-end-labeled double-stranded p53CON, 20 μg of nuclear extract, 2 μg of sonicated salmon sperm DNA, and 100 ng of the anti-p53 mouse monoclonal antibody PAb421 to enhance DNA binding activity as described previously (5, 59). Samples were incubated for 30 min at room temperature and then loaded onto a 4% polyacrylamide gel containing Tris borate/EDTA buffer. Electrophoresis was carried out at 4 °C and 200 V.

RESULTS

Okadaic Acid Induces Hyperphosphorylation of Endogenous p53—Both the stability and activities of p53 are altered in response to a number of stimuli. To determine if okadaic acid caused covalent modification or stabilization of endogenous p53, the effects of the inhibitor on steady-state levels and phosphorylation were determined. Incubation with 500 nM okadaic acid for 6 h had no effect on the amount of p53 immunoprecipitated from \(^{32}P\) methionine-labeled Balb/c 3T3 cells with monoclonal antibody PAb421. Immunoblotting cell extracts with PAb421 also showed that okadaic acid did not affect the level of p53. Phosphorylation of serine 370 by protein kinase C in vitro reduces the reactivity of PAb240 with p53 (33) and could affect the estimate of p53 levels. Therefore, the same cell lysates were also immunoblotted with a second antibody (PAb240) that is independent of phosphorylation (54, 55). The levels of p53 detected with PAb240 and okadaic acid-treated cells were identical to those observed with PAb421, confirming that there was no detectable effect on p53 levels (data not shown). The fact that there was no change in PAb421 immunoreactivity under conditions where p53 becomes highly phosphorylated (see below) indicates that serine 370 is not a major site phosphorylated in response to okadaic acid.

The effects of okadaic acid on p53 phosphorylation were determined by immunoprecipitation of extracts from cells labeled with \(^{32}P\)-labeled inorganic phosphate. Incubation of Balb/c 3T3 cells with okadaic acid caused a dramatic increase in the phosphorylation of p53 (Fig. 1, upper panel). The \(^{32}P\)-labeled protein of M\(_{r}\) = 53,000 was not present in immunoprecipitates from p53-/- cells or in immunoprecipitates derived from the control PAb419 antibody, confirming that this band was p53. The M\(_{r}\) = 76,000 protein was unrelated to p53 since it was also present in p53-/- cells. Okadaic acid-induced increases in p53 phosphorylation were detectable after 1 h, were nearly maximal after 4 h, and persisted for at least 6 h (Fig. 1, lower panel). These data show that okadaic acid causes phosphorylation of endogenous p53 without changing its steady-state levels.

Okadaic Acid Stimulates the Transcriptional Activity of Endogenous p53—The role of phosphorylation in regulating p53 transcriptional activity has been studied using ectopically expressed p53. To determine the role of phosphorylation in controlling endogenous p53, we determined the effects of okadaic acid on transcription of a p53 reporter gene containing a p53 consensus binding site. Balb/c 3T3, p53-/-, or 175.1 cells were cotransfected with the p53CON-Luc reporter plasmid and the pCMV-lacZ plasmid. Twenty-four h after transfection, the cells were treated with okadaic acid, and lysates were assayed for luciferase activity. Luciferase activity in Balb/c 3T3 cells was increased up to 8-fold by incubation with okadaic acid (Fig. 2A).

Okadaic acid had no effect on the very low luciferase activity derived from p53-/- and 175.1 cells, consistent with the absence of functional p53 in these cell lines. The time course of transcriptional activation by okadaic acid was delayed relative to p53 phosphorylation (compare Fig. 2B with Fig. 1). Only a low level of luciferase activity was obtained with the pGup.PA.8 reporter, which lacks the p53-binding element, and the activity was not affected by okadaic acid.

Previous studies have indicated that phosphorylation of p53 enhances DNA binding activity (33, 44, 59). Therefore, we tested for a correlation between okadaic acid-induced phosphorylation of p53 and DNA binding activity. Balb/c 3T3 and p53-/- cells were treated with okadaic acid, and nuclear extracts were analyzed for binding to a \(^{32}P\)-labeled p53CON oligonucleotide using gel mobility shift assays. There was a detectable level of p53CON binding activity in control cells that was stimulated ~10-fold by okadaic acid treatment of Balb/c 3T3 cells. The increase in DNA binding was maximal by 2 h after the addition of 500 nM okadaic acid, as no further increase was seen at 4 or 6 h (data not shown). Beyond 6 h, DNA binding activity decreased as the cells underwent programmed cell death (see below). No DNA binding activity was detected in...
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Okadaic Acid-induced Apoptosis Does Not Require Transcription or Protein Synthesis—Actinomycin D and cycloheximide were used to test the role of p53-mediated gene transcription in okadaic acid-induced apoptosis. Incubation of Balb/c 3T3 cells with the transcription inhibitor actinomycin D did not reduce okadaic acid-induced apoptosis as determined by cell viability (Fig. 6A) or changes in morphology (data not shown). Interestingly, actinomycin D caused an enhancement of apoptosis relative to okadaic acid alone (compare closed circles and squares). At the concentration used (2 μg/ml), actinomycin D did not have an effect on cell viability by itself. Inhibition of mRNA synthesis by actinomycin D was confirmed by measuring the expression of p21. The expression of p21 is induced by p53 (9, 10) and by treating cells with okadaic acid.3 Treatment of Balb/c 3T3 cells with actinomycin D for 1 h completely blocked the effects of okadaic acid on transcription of p21 mRNA (Fig. 6B, lanes 2 and 3). Treatment of Balb/c 3T3 cells with 15 μg/ml cycloheximide was also without effect on apoptosis (Fig. 6A, inverted triangles) despite a 90% inhibition of protein synthesis, as determined by incorporation of [35S]methionine into trichloroacetic acid-precipitable material (data not shown). These results indicate that induction of apoptosis by okadaic acid in these cells does not require the expression of new protein or mRNA and is independent of the transcriptional activity of p53.

Expression of SV40 Small Tumor Antigen Stimulates p53 Transcriptional Activity without Inducing Apoptosis—To further investigate the relationships among phosphorylation, p53-mediated transcription, and apoptosis, we used SV40 small tumor antigen to specifically inhibit PP2A. Overexpression of small t antigen in mammalian cells causes suppression of phoshatase activity by binding to PP2A (50). Balb/c 3T3 cells were cotransfected with small t antigen expression vectors, either the p53CON-Luc reporter or the control pGuP.PA.8 plasmid, and the β-galactosidase expression plasmid. Expression of wild-type small t antigen (SV40-t) caused a 3.7-fold increase in luciferase activity from the p53CON reporter or the control pGuP.PA.8 plasmid, and the β-galactosidase expression plasmid. Expression of wild-type small t antigen (SV40-t) caused a 3.7-fold increase in luciferase expression from the p53CON reporter, but had no effect on the control plasmid (Fig. 7). A control small t antigen plasmid, expressing a small t antigen mutant (SV40-Δt), that is incapable of binding to PP2A, had no effect on luciferase expression. Analysis of cell extracts by immunoblotting showed that equivalent amounts of wild-type and mutant small t antigens were expressed (Fig. 7, inset). This result shows that the

p53-deficient cells.

Okadaic Acid Induces p53-dependent Programmed Cell Death—Okadaic acid caused a dose-dependent decrease in the viability of wild-type Balb/c 3T3 cells (Fig. 3). Under the same conditions, the viability of either p53+/− or 175.1 cells was largely unaffected. To determine if the okadaic acid-induced decrease in Balb/c 3T3 viability was due to programmed cell death, we examined these cells for the appearance of DNA fragmentation, a hallmark of apoptosis (60, 61). Genomic DNA was isolated from all three cell lines cells before and after okadaic acid treatment and analyzed by gel electrophoresis. Treatment of Balb/c 3T3 cells with okadaic acid resulted in a pattern of DNA fragmentation indicative of apoptosis (Fig. 4, upper panel). In contrast, no DNA fragmentation was observed in the p53-deficient cells. DNA fragmentation in Balb/c 3T3 cells appeared 4–6 h after okadaic acid addition and became more apparent with longer treatments (Fig. 4, lower panel). Okadaic acid also induced changes in cell morphology that were dependent on functional p53. Addition of 500 nM okadaic acid to adherent Balb/c 3T3 monolayers caused the cells to round up and detach from the substratum within 3–4 h (Fig. 5A). In contrast, the morphology of p53+/− (Fig. 5B) and 175.1 (Fig. 5C) cells was not altered by 500 nM okadaic acid, even after 6 h. The striking resistance of the p53-deficient cell lines to okadaic acid shows that this phosphatase inhibitor induces rapid, p53-dependent apoptosis.

Okadaic Acid Activity was determined. Relative luciferase activity is expressed as the mean ± S.E. of three independent experiments. B, Balb/c 3T3 cells were cotransfected with pCMV-lacZ and either p53CON-Luc (circles) or the control pGuP.PA.8 plasmid (squares). Twenty-four h later, the cells were treated with 500 nM okadaic acid for the times indicated, and luciferase activity was determined as described for A. The average of two independent experiments is shown.

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3 Y. Yan and M. C. Mumby, unpublished data.
effect of small t antigen on p53 transcriptional activity requires binding to PP2A. Expression of wild-type or mutant small t antigens had no effect on cell viability and did not induce other signs of apoptosis (data not shown). Therefore, specific inhibition of PP2A was sufficient to stimulate p53 transcriptional activity, but was not capable of inducing apoptosis.

**DISCUSSION**

This study shows that inhibition of protein-serine/threonine phosphatases by okadaic acid and SV40 small tumor antigen induces phosphorylation and activation of endogenous p53 in Balb/c 3T3 cells. These results are consistent with previous studies showing that okadaic acid induces hyperphosphorylation of p53 in vitro (42) and in cells that express ectopic p53 (43, 44). The okadaic acid effects indicate that PP1 or PP2A is involved either in the direct dephosphorylation of p53 or in negative regulation of signaling pathways that activate p53. The effects of SV40 small t antigen show that specific inhibition of PP2A is sufficient to cause activation of transcriptional activity. Since dephosphorylation of p53 by PP2A in vitro is inhibited by SV40 small tumor antigen (41), PP2A could be a major p53 phosphatase in vivo.

Treating cells with okadaic acid stimulates phosphorylation of multiple sites within two functional domains of p53. Sites phosphorylated include serines 4, 6, and 15 (based on the numbering of murine p53), within the N-terminal transcriptional activation domain, and serines 309 and 386, within the C-terminal domain involved in oligomerization and regulation of DNA binding activity. The okadaic acid-sensitive phosphorylation sites are conserved in mouse, rat, and human p53 and have all been shown to be phosphorylated in vivo (40). The increases in transcriptional activity in response to okadaic...
Okadaic acid-induced apoptosis is independent of the synthesis of new RNA or protein. A, Balb/c 3T3 cells were treated with either 15 μg/ml cycloheximide (inverted triangles) or 2 μg/ml actinomycin D (circles) or were left untreated (squares) for 1 h. Okadaic acid (500 nM) was added to one set of cells (closed symbols) and omitted from another (open symbols). Viability was then determined at the times indicated by trypan blue exclusion. The values shown represent the average of two independent experiments. B, Balb/c 3T3 cells were incubated for 1 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 2 μg/ml actinomycin D. Either 500 nM okadaic acid (lanes 2 and 3) or control MeSO vehicle (lanes 1 and 4) was added, and the cells were incubated an additional 5 h. Poly(A)^+ RNA was isolated, and 3-μg samples were analyzed by agarose gel electrophoresis and Northern hybridization using a p21 probe. The same blot was also hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to confirm equal loading of mRNA samples.

Okadaic acid, and possibly small tumor antigen, are therefore likely due to both enhanced transactivation and increased DNA binding. These results, obtained with endogenous p53, are consistent with data from several laboratories showing that phosphorylation of p53 is associated with increases in both DNA binding (38, 43, 44, 62) and transcriptional (36, 39, 62) activities. In contrast, a previous report using the human K-562 leukemia cell line, which lacks endogenous p53, showed that okadaic acid decreased the transcriptional activity of ectopically expressed p53 (43). We have observed a similar okadaic acid-induced decrease in p53 transcriptional activity when p53 is ectopically expressed in human lung carcinoma H-1299 cells, which lack functional p53 (43). While the reasons for these differences are not known, they indicate that results obtained with overexpressed p53 should be interpreted with caution.

The data presented here show that okadaic acid induces p53-dependent apoptosis. The fact that apoptosis is not prevented by cycloheximide or actinomycin D demonstrates that the transcriptive activity of p53 is not required for this effect. A lack of involvement of p53-regulated transcription is also supported by the observation that SV40 small tumor antigen stimulates transcriptional activity, but does not cause apoptosis. Balb/c 3T3 cells lacking functional p53 or expressing a dominant-negative p53 mutant have a striking resistance to okadaic acid-induced apoptosis. Previous studies showed that okadaic acid causes programmed cell death in many primary cells and tumor cell lines (reviewed in Ref. 47). High concentrations (0.1–1.0 μM) of okadaic acid cause rapid apoptosis (45), and lower concentrations (5–20 nM) of either okadaic acid or calyculin A, a related phosphatase inhibitor, induce apoptosis with a delayed time course (46). Interestingly, AU-565 breast tumor cells are resistant to the apoptotic effects of both phosphatase inhibitors. Other cell lines reported to be resistant to okadaic acid include HT29, SCL209, and DC3F (63). The p53 status of these okadaic acid-resistant cells is not known. Our results support the conclusions that induction of apoptosis by okadaic acid requires p53 and occurs through pathways that do not involve p53 transactivation (47).

p53-dependent apoptosis can occur in the absence of transcripcional activity under some conditions. This has been shown using RNA and protein synthesis inhibitors (18, 19) and with mutants of p53 deficient in sequence-specific transcription activity (20). In contrast, the transcriptional activity of p53 appears to be required under other conditions, including induction of apoptosis by E1A (64). It has therefore been postulated that p53-dependent apoptosis is mediated by multiple pathways, one of which depends on its transactivating function (20, 65). The nature of the transcription-independent pathway is not known, but may involve the transcriptional repressor activity of p53 (20, 65, 66). Consistent with this idea are the observations that phosphorylation at the casein kinase 2 site (serine 386) is associated with increases in transcriptional repressor activity (67) and that phosphorylation of this site is increased by okadaic acid (44). The results reported here show that inhibition of PP1 or PP2A by okadaic acid is another circumstance where p53 is required for apoptosis, but its action is independent of transactivation.

While phosphorylation has been implicated in the control of the transcriptional and growth suppressor activities of p53 (40, 68), its role in p53-mediated apoptosis has not been addressed. The time course of okadaic acid-induced phosphorylation of p53 (Fig. 1) precedes the appearance of DNA fragmentation (Fig. 4). These observations are consistent with a role for phosphorylation in p53-dependent apoptosis. However, since okadaic acid inhibits the dephosphorylation of a wide spectrum of proteins, induction of apoptosis could also be due to increased phosphorylation of other proteins that require p53 function to cause apoptosis. Whether the effect of okadaic acid involves direct changes in p53 phosphorylation or not, our data show that disruption of signaling pathways that utilize phosphorylation and dephosphorylation has dramatic effects on p53 function.

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