Effect of Extracellular Signal-regulated Kinase on p53 Accumulation in Response to Cisplatin*

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Received for publication, May 18, 2000, and in revised form, July 24, 2000. Published, JBC Papers in Press, August 24, 2000, DOI 10.1074/jbc.M004267200

The p53 tumor suppressor protein is a transcription factor that plays a major role in the DNA damage response. After DNA damage, p53 levels increase due primarily to stabilization of the protein. The molecular mechanisms leading to stabilization of p53 after DNA damage have not been completely elucidated. Recently we reported that cisplatin treatment activated extracellular signal-regulated kinase 1 and 2 (ERK1/2) and that inhibition of ERK1/2 resulted in enhanced sensitivity to cisplatin. In the present study, we examined the potential role of ERK1/2 activation in regulation of the p53 response to cisplatin. In the ovarian carcinoma cell line A2780, inhibition of ERK1/2 activation with the mitogen-activated protein kinase/ERK kinase 1 (MEK1) inhibitor PD98059 resulted in decreased p53 protein half-life and diminished accumulation of p53 protein during exposure to cisplatin. We also demonstrated that p53 protein co-immunoprecipitated with ERK1/2 protein and was phosphorylated by activated recombinant murine ERK2 in vitro. Furthermore, PD98059 decreased the phosphorylation of p53 at serine 15 during cisplatin exposure, suggesting that ERK1/2 mediates in part phosphorylation of p53 during the cisplatin DNA response. These results strongly suggest that cisplatin-induced ERK activation is an up-stream regulator of the p53 response to DNA damage caused by cisplatin.

The p53 protein is normally rapidly degraded by ubiquitin-dependent proteolysis, a process that is regulated primarily by binding of MDM2 protein to p53 (14–16). MDM2 binding to the N terminus of p53 mediates the ubiquitin proteosome degradation of p53 and also regulates transactivation of p53 (15, 16). In response to DNA damage, the amount of p53 protein is elevated primarily due to increased protein half-life (17). The exact mechanism by which DNA damage affects p53 protein stability remains to be completely defined. However, post-translational modifications, including DNA damage-induced phosphorylation of the N terminus of p53, have been associated with regulation of p53 stability (18, 19). Specifically, phosphorylation of p53 at serine 15 (near the MDM2 binding domain) has been shown to weaken the association between MDM2 and p53 (18). Therefore, up-stream kinases that are involved in phosphorylation of p53 at sites near the MDM2 binding domain may participate in MDM2-dependent regulation of p53 accumulation after DNA damage.

Several members of the phosphatidylinositol 3-kinase family, namely DNA-activated protein kinase (20), ataxia telangiectasia mutated (ATM) kinase (21, 22), and the ATM-Rad3-related (ATR) kinase (23, 24), are capable of phosphorylating p53 at serine 15 in vitro. Among other protein kinases that have been shown to phosphorylate p53 are the mitogen-activated protein (MAP) kinases (25–29). The MAP kinase pathways are parallel cascades of structurally related serine/threonine kinases that serve to integrate numerous extracellular signals, resulting in regulation of cell proliferation, differentiation, and cell survival (30–34). Extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase, and p38 are the terminal enzymes in three major kinase cascades within the MAP kinase family (31, 32, 35). Two members of the MAP kinases have recently been shown to interact with p53. Fuchs et al. (28) demonstrate that c-Jun-N-terminal kinase activation via MAPK kinase-1 (MEKK1) results in p53 phosphorylation, inhibition of MDM2 association with p53, and an increase in p53 protein half-life. In addition, non-activated c-Jun-N-terminal kinase has been shown to target p53 ubiquitination and degradation in nonstressed cells (29). Recently, the MAP kinase p38 also was shown to phosphorylate p53 in response to ultraviolet (UV) irradiation and to be required for UV-induced p53-dependent transcription (26, 27, 36). In contrast, ERK1/2 interaction with human p53 has not been previously described. Although in vitro phosphorylation of mouse p53 by activated recombinant ERK has been reported, the corresponding residues in human p53 are not conserved serine/threonine phosphorylation sites (37, 38).

We recently reported that cisplatin induces ERK1/2 activation in ovarian carcinoma cell lines (39). In this study, we sought to determine whether the p53 response to cisplatin DNA damage was influenced by cisplatin-induced ERK1/2 activation. We demonstrated that inhibition of cisplatin-induced...
ERK1/2 activity by the MAP/ERK kinase 1 (MEK1) inhibitor PD98059 decreased p53 protein half-life and accumulation during cisplatin treatment in the ovarian carcinoma cell line A2780. Furthermore, we showed that cisplatin-induced phosphorylation of p53 at serine 15 is mediated in part by ERK1/2. These results strongly suggest that ERK1/2 is one of the regulators of p53 protein accumulation and activation during the DNA damage response to cisplatin.

**MATERIALS AND METHODS**

**Cell Line and Preparation of Cellular Extracts**—The human ovarian carcinoma cell line A2780 (a gift from Dr. Thomas C. Hamilton, Dept. of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania) was maintained in RPMI 1640 medium containing penicillin/streptomycin/glutamine, 0.3 unit/ml insulin (Life Technologies, Inc.), and 10% fetal bovine serum (Intergen, Purchase, NY) in a humidified atmosphere of 5% CO₂ at 37 °C. For the dose-response and the time-course studies, cells were grown to 70–80% confluency in 100-mm Petri dishes for 72 h. Cells were then treated for 24 h with different concentrations of cis-platinum(II)dimethylglycolurate (cisplatin, Sigma) dissolved in RPMI 1640 medium or with 10 μg/ml cisplatin for varying periods of time. In studies using kinase or proteosome inhibitors, cells were pretreated with the appropriate inhibitor as follows: 1 h with 100 μM PD98059 (Calbiochem-Novabiochem) dissolved in Me₂SO, 1 h with 10 μM SB202190 (Calbiochem-Novabiochem) dissolved in Me₂SO, 30 min with 200 μM wortmannin (Sigma) dissolved in Me₂SO, 15 min with 2.5 mM caffeine (Sigma) dissolved in H₂O:Me₂SO (3:2, v:v), or 2 h with 20 μM calpain/proteosome inhibitor LLA LL (Sigma) dissolved in Me₂SO. Cells were also continuously exposed to the inhibitors during the exposure to cisplatin. After treatment, cells were washed with phosphate-buffered saline and lysed in 500 μl of ice-cold Triton X-100 lysis buffer (TLB: 20 mM Tris, pH 7.4, with 137 mM NaCl, 25 mM glycerophosphate, 2 mM EDTA, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1% Triton X-100, and 10% glycerol) with protease inhibitors (TLB: TLB + TLB with 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 2 μg/ml aprotinin). Cell extracts were clarified by centrifugation at 13,000 × g for 15 min. Portions of the same cellular extracts were used for ERK1/2 immune complex kinase assays, immunoprecipitation studies, and Western blot analysis.

**ERK1/2 Immune Complex Kinase Assay—**ERK1/2 was immunoprecipitated from 200 μg of total protein extracted from A2780 cells with anti-ERK1 and anti-ERK2 antibodies (C-16 and C-14, Santa Cruz Biotechnology) bound to Protein A/G Plus-agarose for 2 h at 4 °C. Immune complex kinase assays were performed in a final volume of 28 μl of ERK assay buffer (40 mM Tris, pH 7.4, 20 mM MgCl₂, 2 mM MnCl₂, 0.5 mM DTT) with 11 μg of myelin basic protein (Sigma) as a substrate, 30 μM unlabeled ATP, and 9 μCi of [γ-³²P]ATP (Amersham Pharmacia Biotech). Reactions were conducted for 30 min at 30 °C and terminated by the addition of 2× SDS-PAGE sample buffer. Samples were resolved by 14% SDS-PAGE (Novex), stained, dried, and autoradiographed. The kinase activity was quantified using PhosphorImager analysis.

In vitro kinase assays using recombinant ERK2 were performed in a final volume of 20 μl of buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 2 mM EGTA, 2 mM DTT, 0.01% Brij, New England Biolabs) with different amounts of (50, 10, or 1 unit) of activated mouse ERK2 (New England Biolabs), 9 μCi of [γ-³²P]ATP, and either human recombinant glutathione S-transferase-p53 (5 or 1 μg, Santa Cruz Biotechnology) or p53 immunoprecipitated from A2780 cells. Reactions were conducted for 30 min at 30 °C and terminated by the addition of 2× SDS-PAGE sample buffer. Samples were resolved using 14% SDS-PAGE, stained, dried, and autoradiographed. The kinase activity was quantified using PhosphorImager analysis.

**Co-immunoprecipitation Studies**—For co-immunoprecipitation studies, anti-ERK1 and anti-ERK2 (C-16 and C-14, Santa Cruz Biotechnology) antibodies were coupled to Sepharose as described by Harlow and Lane (40). Briefly, antibody was bound to protein A-Sepharose (Amer sham Pharmacia Biotech) for 1 h at room temperature with rotating. The protein A-Sepharose–antibody complex was washed twice and re-suspended in 200 mM sodium borate, pH 9.0. Dimethyl pimelimidate (20 mM final concentration, Sigma) was added to the mixture and rotated for 30 min at room temperature. The coupling reaction was stopped by washing the beads with 10 volumes of 200 mM ethanolamine, pH 8.0, followed by further washing with 200 mM ethanolamine, pH 8.0, for an additional 2 h at room temperature with gentle mixing. Coupled antibodies were stored in the dark at 4 °C in phosphate-buffered saline containing 0.01% merthiolate (Sigma) as 50% slurry. The above-coupled antibodies (40 μl of a 50% slurry) were then used to immunoprecipitate ERK1/2 proteins from 200 μg of total cellular protein extracts in 500 μl of TLB + without DTT. Immunoprecipitation was carried out at 4 °C for 2 h with rotation, after which the bound immune complexes were washed twice with TLB + without DTT, resuspended in 30 μl of 2× SDS-PAGE sample buffer, heated at 95 °C for 10 min, cleared by centrifugation, and subjected to Western blot analysis.

Western Blot Analysis—Cellular extracts (20 μg of total protein) were resolved using 12% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk, washed, and incubated with the following primary antibodies: anti-p53 (DO-1, Santa Cruz Biotechnology), anti-p21WAF-1 (C-19, Santa Cruz Biotechnology), anti-MDM2 (SMP14, Sanat Cruz Biotechnology), anti-phospho-p53 serine 15 (New England Biolabs, Beverly, MA), or anti-ERK1 and anti-ERK2 (C-16 and C-14, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibody (1:10,000, Sigma) was used to detect the bound primary antibody. Immune complexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) following the manufacturer’s instructions. The intensity of the bands was quantitated by densitometry (Personal Densitometer, Molecular Dynamics).

**p53 Half-life Studies**—Cells were grown in 100-mm Petri dishes for 72 h to 70–80% confluency. Cells were pretreated for 1 h with 100 μM PD98059 dissolved in Me₂SO and then were treated with 10 μg/ml cisplatin for 8 h. After treatment, cells were incubated with 20 μg/ml cycloheximide for various periods of time, washed with phosphate-buffered saline, and lysed in 500 μl of ice-cold TLB +. Cellular extracts (20 μg of total protein) from each time point were subjected to immunoblotting using anti-p53 antibody. The intensity of the bands was determined by densitometry.

**RESULTS**

Cisplatin Induction of ERK1/2 Activity—A dose-dependent activation of ERK1/2 in A2780 cells, as measured by an immune complex kinase assay, was observed as illustrated in Fig. 1A. ERK1/2 activation peaked after 24 h of exposure to 10 μg/ml cisplatin (5.7-fold induction above control). The ERK1/2 activation during exposure to cisplatin was due to an increase in kinase activity and not secondary to elevation of ERK protein levels, as Western blot analysis of ERK1/2 proteins showed no significant changes in protein levels when ERK1/2 activity was increased (Fig. 1A, lower blot).
Inhibition of cisplatin-induced ERK1/2 activity by PD98059. ERK1/2 immune complex kinase assays were performed using protein extracts from A2780 cells pretreated for 1 h with or without 100 μM PD98059 or the solvent MeSO (DMSO), followed by treatment with or without 10 μg/ml cisplatin for 24 h. Fold activation of ERK1/2 was calculated as the cpm ratio of treated samples to the untreated sample by PhosphorImager analysis.

In time-course studies, ERK1/2 activation increased above baseline level at 8 h after exposure to 10 μg/ml cisplatin (Fig. 1B, top gel), peaked at 18 to 24 h, and decreased significantly by 48 h (48-h data not shown). No significant changes in ERK1/2 protein levels occurred over the corresponding time course (Fig. 1B, bottom gel). These results differ from those reported by Sanchez-Perez et al. (41) in which significant induction of ERK1/2, as measured by the change in mobility shift on Western blot analysis, was observed in Pam 212 cells (41). The time course of these investigators, however, extended only up to 9 h, and it is possible that induction may have been observed at later times, similar to our results. The delayed induction of ERK1/2 in our investigation was noteworthy, since induction of ERK1/2 activity by other stimuli such as epidermal growth factor and the tumor-promoting phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), has been shown to occur at earlier times, usually within less than an hour after exposure (42).

Because cisplatin-induced DNA damage caused by formation of cisplatin-DNA adducts may require several hours to occur, the relatively late activation of ERK1/2 correlates with it being a downstream event of cisplatin-induced DNA damage.

The cisplatin-induced ERK1/2 activation can be readily inhibited by the use of PD98059, a synthetic inhibitor of MEK1 (the immediate upstream kinase of ERK1/2). PD98059 inhibits phosphorylation and activation of MEK1 (43, 44), thereby inhibiting phosphorylation and activation of ERK1/2. PD98059 caused a complete inhibition of the cisplatin-induced ERK1/2 activation and had little effect on untreated A2780 cells (Fig. 2).

Inhibition of Cisplatin-induced ERK1/2 Activity and Decreased p53 Protein Accumulation—Two of the major MAP kinases, c-Jun-N-terminal kinase and p38, have been shown to interact with p53 protein, resulting in changes in p53 stability or activity (26–29). Therefore, we examined whether inhibition of cisplatin-DNA damage (45–47). The accumulation of p53 protein after DNA damage caused by UV irradiation and γ-irradiation has been shown to be due to a prolonged half-life of the protein (17). A dose-dependent increase in p53 protein accumulation after cisplatin-induced DNA damage was observed in A2780 cells (Fig. 3A). As with ERK1/2 activation, the peak level of p53 accumulation occurred with 10 μg/ml cisplatin. Therefore, this concentration was used for the time-course studies with p53 and for subsequent studies using cisplatin.

The time course of cisplatin-induced accumulation of p53 protein levels in A2780 cells is illustrated in Fig. 3B. A slight increase in the relative p53 protein level was observed at 4 and 8 h. However, the most dramatic increase in accumulation did not occur until 12–18 h after initiation of cisplatin treatment. This time course is consistent with our hypothesis that ERK activation contributes to p53 stabilization and accumulation after cisplatin treatment. The fact that there is a small amount of p53 accumulation observed at 4 h and ERK activation is not readily detected until 8 h suggests that other factors also contribute to stabilization of p53. Treatment of cells with the combination of PD98059 and cisplatin resulted in a significant reduction in cisplatin-induced p53 accumulation at 12, 18, 24, and 28 h. Inhibition of ERK activation by PD98059 in cells without cisplatin resulted in minimal changes in p53 protein levels. Although PD98059 did not completely abolish p53 accumulation, the results suggest that ERK activity may significantly contribute to p53 accumulation during cisplatin exposure. Similar inhibition of p53 accumulation after treatment with PD98059 and cisplatin was observed in the breast cancer cell line MCF-7, indicating that this finding was not a cell line-specific phenomenon (data not shown).

Inhibition of Cisplatin-induced ERK1/2 Activity and Decreased p53 Protein Half-life—In view of the fact that ERK1/2 activation affected p53 protein accumulation during cisplatin exposure, we next examined the effect of cisplatin-induced ERK activity on p53 protein half-life. Cells were treated with 10 μg/ml cisplatin in the presence or absence of PD98059 for 8 h, and then cycloheximide (20 μg/ml) was added to the cultures to inhibit protein synthesis (Fig. 4). The relative amounts of p53 protein remaining at various time points after cycloheximide treatment were then measured after Western blot analysis using densitometry. Cisplatin caused a prolongation of p53 protein half-life from approximately 2 h in untreated cells to 8 h in cisplatin-treated cells. Inhibition of ERK1/2 activity by PD98059 in cells treated with cisplatin resulted in a significant decrease in p53 protein half-life (8 h with cisplatin compared with approximately 3 h with PD98059 and cisplatin). PD98059 had no effect on p53 half-life in the absence of cisplatin treatment. To verify that the MEK1 inhibitor PD98059 had no effect by itself on protein half-life, similar half-life studies were performed on p21WAF-1 protein. PD98059 had no effect on p21WAF-1 half-life before or after cisplatin treatment (data not shown).

Co-immunoprecipitation of p53 Protein with ERK1/2 Protein—Given the fact that inhibition of cisplatin-induced ERK activation led to a decrease in p53 protein half-life and a decrease in p53 protein accumulation, we next examined the possibility that ERK1/2 protein interacted directly with p53 protein. Immunoprecipitation of whole cell extracts with anti-ERK1 and anti-ERK2 antibodies followed by immunoblotting with anti-p53 antibodies demonstrated that p53 protein was co-immunoprecipitated with ERK1/2 protein (Fig. 5). Likewise, immunoprecipitation with an antibody to p53 protein followed by immunoblotting with antibodies to ERK1/2 proteins also showed that ERK1/2 co-immunoprecipitated with p53 (data not shown). Although small amounts of p53 protein co-immunoprecipitated with ERK1/2 in untreated cells, the amount of p53 that was associated with activated ERK1/2 in cisplatin-treated cells was higher. In addition, inhibition of cisplatin-induced ERK1/2 activation by PD98059 resulted in a decreased amount of p53 protein that was associated with immunoprecipitated ERK1/2. The difference in the amount of co-immunoprecipitated p53 protein may be due to either higher levels of p53 protein being available for binding to ERK1/2 after exposure to cisplatin or to preferential binding between cisplatin-activated p53 and ERK1/2 proteins. Phosphorylation of Human p53 by Activated Recombinant ERK—To further investigate the significance of the interaction between ERK1/2 and p53 proteins, we examined the potential of ERK to phosphorylate human p53 protein. Milne et al. (37) previously reported that recombinant wild-type mouse p53 was phosphorylated at threonine residues 73 and 83 in vitro by...
activated mouse recombinant ERK. However, the corresponding residues are not conserved threonine or serine sites in human p53 (38). To the best of our knowledge, phosphorylation of human p53 by ERK has not been reported. Therefore, we examined whether human p53 protein could also be phosphorylated by activated ERK in vitro. Fig. 6A illustrates the results of an in vitro ERK kinase assay utilizing recombinant human glutathione S-transferase-p53 protein as the substrate, in which increasing levels of phosphorylation were achieved with increasing concentrations of activated recombinant ERK2. These studies demonstrate that human p53 protein can be phosphorylated by activated ERK2 in vitro. In addition, we demonstrated that immunoprecipitated p53 from A2780 cells treated with cisplatin could also be phosphorylated by activated ERK (Fig. 6B).

Effect of PD98059 on Phosphorylation of p53 at Serine 15—Phosphorylation of p53 at serine 15 has been shown to impair the ability of MDM2 to interact with p53, thereby providing a potential mechanism for regulation of p53 stability (18, 48). Western blot analysis was performed using anti-phospho-p53 serine 15 antibody to determine if ERK1/2 activation affected the relative level of phosphorylation of p53 at serine 15. Fig. 7A illustrates that cisplatin induced the phosphorylation of p53 at serine 15 (bottom gel, third lane from the left). By comparing the amount of total p53 protein (top gel) to the amount of p53 specifically phosphorylated at serine 15, the relative amount of phosphorylation at serine 15 was determined. Although 72% of the total amount of p53 protein was phosphorylated at serine
15 during cisplatin treatment, only 19% of total p53 protein remaining after cisplatin, and PD98059 treatment was phosphorylated at serine 15. To control for changes in p53 protein accumulation due to degradation upon cisplatin treatment and to allow for comparison of comparable levels of p53 phosphorylated with or without cisplatin and PD98059, cells were pretreated with the calpain/proteasome inhibitor, LLaL, as shown in Fig. 7B (49). We observed a significant decrease in the percent of total p53 that was phosphorylated at serine 15 after treatment with PD98059. These findings provide evidence that cisplatin-induced ERK1/2 activation contributed to phosphorylation of p53 at serine 15 during cisplatin exposure. Because phosphorylation of p53 at serine 15 can reduce MDM2 binding and thus interfere with p53 degradation, this may be a mechanism by which ERK activation contributes to the accumulation of p53 protein.

Effect of SB202190, Wortmannin, and Caffeine on Phosphorylation of p53 at Serine 15—Several other kinases including DNA-activated protein kinase, ATM, ATR, and p38 have been shown to be capable of phosphorylating p53 at serine 15 after DNA damage by various agents such as UV and γ-irradiation (20–24). However, the kinases involved in phosphorylating p53 after cisplatin DNA damage have not been examined. Therefore, compounds that have been shown to inhibit p38 (SB202190), DNA-activated protein kinase (wortmannin), ATM (wortmannin and caffeine), and ATR (caffeine) were examined for their effect either independently or in combination with PD98059 on total p53 accumulation and phosphorylation of p53 at serine 15 during cisplatin exposure (50–52). As illustrated in Fig. 8A and B, SB202190 caused a slight increase in the total amount of p53 protein (Fig. 8B, left) but did not have a significant effect on the ratio of p53 phosphorylated at serine 15 to total p53 (Fig. 8B, right). The combination of PD98059 and SB202190 resulted in a decrease in total p53 but had little effect on the ratio of p53 phosphorylated at serine 15 to total p53. In contrast, both wortmannin and caffeine decreased the total amount of p53 protein that accumulated during the cisplatin exposure, suggesting that both wortmannin- and caffeine-sensitive pathways also mediate cisplatin-induced p53 accumulation. Although the total amount of p53 phosphorylated at serine 15 was decreased by both wortmannin and caffeine (Fig. 8B, center), this apparent effect on serine 15 phosphorylation was in fact due to the decrease in total p53 levels. PD98059 was the only inhibitor that decreased the ratio of p53 phosphorylated at serine 15 to total p53. Wortmannin had little effect on the percent of total p53 that was phosphorylated at serine 15, and caffeine appeared to enhance the proportion of serine 15 phosphorylated p53.

Effect of Cisplatin-induced ERK Inhibition on p53 Function—Accumulation and activation of p53 protein after DNA damage can mediate a number of different cellular responses, in part through transcriptional activation of numerous genes (4–13). Many of these cellular responses may be critical in determining sensitivity to cisplatin. We examined the effect of PD98059 on expression of two p53-targeted genes, p21\textsuperscript{WAF1} and MDM2. Fig. 9 illustrates that both p21\textsuperscript{WAF1} and MDM2 protein levels increased in response to cisplatin, and the accumulation of both proteins was inhibited by PD98059. Therefore, after combined treatment of cisplatin and PD98059, the transactivation function of p53 protein, as measured by transcriptional activation of p21\textsuperscript{WAF1} and MDM2, was decreased and correlated with the decreased p53 accumulation.

DISCUSSION

The p53 tumor suppressor protein is activated and stabilized in response to several cellular stresses including DNA damage (53). The regulation of p53 activation and stabilization after DNA damage involves protein-protein association, protein turnover, and post-translational modifications (54). Central to the regulation of p53 is the binding of MDM2 protein to p53, which results in blocking of p53-transactivating function and targeting of p53 for ubiquitin-mediated degradation (14–16). Several reports have linked phosphorylation of N-terminal residues of p53 protein with its activation and stability (18, 19), suggesting that phosphorylation of residues near the MDM2 binding region (residues 18–23) of p53 may be a mechanism by which interaction of the two proteins is prevented. It is well established that cellular stress leads to phosphorylation of p53 at multiple sites clustered on the N-terminal and C-terminal regions of p53 (25). Shieh et al. (18) report that DNA damage-induced phosphorylation of p53 at serine 15 weakens both the p53 association with MDM2 and the ability of MDM2 to inhibit the activity of p53 (18). However, other investigators demonstrate that phosphorylation of serine 15 has only a weak, if any, effect on MDM2 binding \textit{in vitro} (55, 56). Therefore, the extent to which phosphorylation is required for the dissociation of the p53-MDM2 complex remains to be determined. The phosphorylation of serine 15 after DNA damage has also been shown to have a potential role in activation of p53. Phosphorylation of p53 at serine 15 increases its ability to recruit CREB (cAMP-response element-binding protein)-binding protein (CBP)/p300, which in turn, through interaction with the N terminus of p53, increases the sequence-specific DNA binding activity of p53 (57). Thus, kinases that phosphorylate p53 at serine 15 after DNA damage are potentially important upstream regulators of the p53 response to DNA damage.

We have demonstrated that inhibition of cisplatin-induced ERK1/2 activation decreases the level of p53 accumulation during cisplatin treatment and that the decreased accumulation is secondary to a shorter half-life of the protein. The decreased accumulation of p53 protein also resulted in decreased function of p53, as demonstrated by lower levels of the p53-dependent p21\textsuperscript{WAF1} and MDM2 proteins after inhibition of ERK1/2 activity. These findings indicate that ERK1/2 activa-
tion is required for maximum accumulation and activation of p53 after DNA damage caused by cisplatin. However, because PD98059 does not completely inhibit accumulation of p53 protein, additional up-stream signaling pathways must also contribute the stability of p53 after cisplatin-induced DNA damage.

The mechanism by which ERK1/2 activation contributes to p53 accumulation may be through its ability to phosphorylate p53 at serine 15, thereby disrupting the p53-MDM2 complex. Recently, other members of the MAP kinase family, namely c-Jun-N-terminal kinase and p38, have also been shown to phosphorylate p53 protein (31, 32, 35, 36). Activated c-Jun-N-terminal kinase phosphorylates mouse p53 at serine 34 and has been shown to affect MDM2 binding to p53 (28). The p38
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MAP kinase mediates UV-induced phosphorylation of p53 protein at serine 389 and has also been associated with UV-induced activation of p53. Recently, She et al. (36) report that p38 and ERK1/2 mediate UVB-induced phosphorylation of mouse p53 at serine 15. Although previous studies also demonstrate that activated recombinant ERK can phosphorylate mouse p53 in vitro at two N-terminal sites, threonine residues 73 and 83, the corresponding residues in human p53 are not conserved sites for serine/threonine phosphorylation (37, 38). Thus, our studies are the first to show that human p53 is phosphorylated by ERK1/2 after DNA damage.

In addition to the recently reported involvement of p38 in phosphorylation of p53 at serine 15, three other kinases, all members of the phosphatidylinositol 3-kinase family, have been reported to phosphorylate p53 at serine 15 in vivo: DNA-activated protein kinase (20), ATM (21, 22), and ATR (23, 24). Our observation that ERK1/2 was only partially responsible for serine 15 phosphorylation during cisplatin exposure suggested that multiple kinases were involved in this post-translational modification. To further examine this possibility, we used selective inhibitors for DNA-activated protein kinase (wortmannin), ATM (wortmannin, caffeine), ATR (caffeine), and p38 (SB202190) and determined that p53 accumulation after cisplatin treatment was also dependent on wortmannin- and caffeine-sensitive pathways. However, the relative amount of p53 phosphorylated at serine 15 compared with total p53 was not significantly altered by wortmannin or caffeine. These findings suggest that DNA-activated protein kinase, ATM, or ATR may play a role in increasing the stability of p53 protein during cisplatin exposure, but the mechanism by which this occurs appears to be independent of phosphorylation of p53 at serine 15. The inhibitor for the MAP kinase p38, SB202190, had the opposite effect on p53 accumulation, slightly enhancing the total amount of p53 protein. Similar to wortmannin and caffeine, the p38 kinase inhibitor SB202190 did not appear to affect the phosphorylation of p53 at serine 15 during cisplatin exposure.

Our results support the hypothesis that different upstream signal transducers activate p53 in response to different forms of DNA damage. The ATM kinase has been implicated as the prime candidate for the protein kinase that phosphorylates p53 at serine 15 in response to ionizing radiation (21–23). ATR, which phosphorylates both serine 15 and serine 37, has been shown to play a central role in the activation of p53 by UV irradiation (24). In addition, both p38 and ERK1/2 have also recently been shown to have a direct role in UV-induced phosphorylation of p53 at serine 15 in mouse JB6 epidermal cells (36). In our studies, the ERK1/2 inhibitor PD98059 was the only selective inhibitor that caused a decrease in the relative level of p53 phosphorylated at serine 15 compared with total p53, suggesting that of the kinases examined in this study, only ERK1/2 contributed to serine 15 phosphorylation. Our data also suggest that a caffeine-sensitive pathway may partially inhibit serine 15 phosphorylation during cisplatin treatment, whereas wortmannin-sensitive pathways and the p38 pathway do not affect phosphorylation at serine 15.

In conclusion, we have identified ERK1/2 as a protein kinase that targets p53 for phosphorylation after cisplatin-induced DNA damage. Our demonstration of the role of ERK1/2 in phosphorylation of p53 at serine 15 suggests a potential mechanism by which ERK1/2 activation may augment p53 protein stability and p53 function as a transcription factor, namely through disruption of the p53-MDM2 complex. This study provides the first evidence that the ERK1/2 pathway is one of the upstream mediators of the p53 DNA damage response after exposure to cisplatin.

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