Regulation of p53 Stabilization by DNA Damage and Protein Kinase C[1]

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Abstract

We have demonstrated previously that the protein kinase C (PKC) signal transduction pathway acts upstream of caspases to regulate caspase activation and apoptosis induced by the DNA-damaging agent cisplatin (CP). In the present study, we have examined whether PKC influences p53 and, hence, cellular sensitivity/resistance to CP. The basal p53 level was low in HeLa cells but was elevated in CP-resistant HeLa (HeLa/CP) cells. CP had no effect on the p53 content in HeLa cells, but it caused p53 accumulation in HeLa/CP cells. Rottlerin, a PKCδ inhibitor that prevents CP-induced proteolytic activation of PKCδ, caused an accumulation of p53 in HeLa cells when treated in conjunction with CP, but it had no additional effect in HeLa/CP cells. The ability of rottlerin to prevent proteolytic activation of PKCδ or to induce accumulation of p53 by CP was compromised in HeLa/CP cells. PKC activator phorbol 12, 13-dibutyrate attenuated constitutive p53 levels in both HeLa and HeLa/CP cells. Whereas the combination of rottlerin and CP increased the half-life of p53 in HeLa cells, CP alone was sufficient to stabilize p53 in HeLa/CP cells. These results suggest that both DNA damage and inhibition of proteolytic activation of PKCδ by CP were necessary for the stabilization of p53 in HeLa cells. Furthermore, an increase in p53 was not associated with enhanced sensitivity of HeLa cells to CP.

Introduction

CP[3] is one of the most effective anticancer drugs used in the treatment of solid tumors, including ovarian, testicular, small cell lung, and cervical carcinomas (1, 2). Although CP therapy is effective in treating solid tumors, its success is often compromised by the development of drug resistance (3). The antitumor activity of CP is believed to be because of its interaction with chromosomal DNA (4). Only a small fraction of CP actually interacts with DNA, and the inhibition of DNA replication cannot solely account for its biological activity (1). The efficacy of chemotherapeutic drugs not only depends on their ability to induce DNA damage but also on the ability of the cell to detect and respond to DNA damage (5).

The tumor suppressor protein p53 is activated in response to DNA damage to protect normal cells by inducing transcription of regulatory genes involved in cell cycle, apoptosis, and DNA repair (6). The *p53* gene is mutated in half of all cancers, and there is often a lack of p53 function in those cases in which *p53* is not mutated (6, 7). The majority of malignant cervical cancer cells, including human cervical carcinoma (HeLa) cells, contain wild-type *p53*. However, the E6 gene product of HPV can interact with *p53*, resulting in the rapid degradation of p53 through the ubiquitin-proteasome pathway (8). Therefore, although HeLa cells contain a wild-type *p53* gene, expression of the E6 protein from high-risk HPV can have the same functional consequences as a mutated *p53* gene.

p53 is regulated primarily through post-translational modifications, especially phosphorylation, and the accumulation of p53 is the first step in response to cellular stress (9). The *HDM2* (human homologue of MDM2) gene is a transcriptional target of *p53*, and once synthesized, the MDM2 protein can bind to p53 at its NH_2 terminus leading to its rapid degradation through the ubiquitin proteasome-mediated pathway (7, 9–11). In response to DNA damage, p53 becomes phosphorylated at multiple sites at the NH_2 terminus, thereby inhibiting MDM2 binding (6, 7, 9–12). As a result, p53 degradation does not occur and p53 accumulates.

p53 can also be phosphorylated at its COOH-terminal regulatory domain, which influences its DNA binding (13). It has been reported recently that constitutive phosphorylation of p53 by PKC at its COOH-terminal domain can lead to its degradation via ubiquitin proteasome-mediated pathway (14). Treatment of mouse or human fibroblasts with PKC inhibitors, such as H7 or bisindolylmaleimide I, inhibited COOH-terminal phosphorylation of p53 and increased accumulation of p53 without affecting the formation of the p53-MDM2 complex (14). However, PKC inhibitors were unable to increase accumulation of p53 in HPV-positive HeLa cells (14, 15).

Previous studies have shown that the PKC signal transduction pathway regulates cell death by CP in HeLa cells (16–20). PKC represents a family of 11 isozymes: conventional PKCs (α, βI, βII, and γ), novel PKCs (δ, ϵ, η, θ, and μ), and atypical PKCs (ζ and λ/ι). Novel PKCδ, -θ, and -μ are substrates for the effector caspase-3, and proteolytic activation of these novel PKCs has been associated with cell death (21–23). However, we have shown that PKC acts upstream of caspases to regulate cell death by CP. For example, PKC activators enhanced CP-induced caspase activa-

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[3] The abbreviations used are: CP, *cis*-diamminedichloroplatinum(II) or cisplatin; HPV, human papillomavirus; PDBu, phorbol 12, 13-dibutyrate; PKC, protein kinase C; Rot, rottlerin; MDM2, murine double minute 2.

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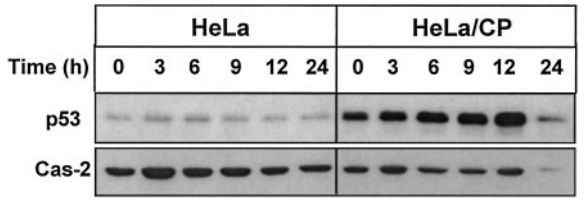

Fig. 1. Effect of CP on p53 levels in parental and CP-resistant HeLa cells. HeLa and HeLa/CP cells were treated with 10 μM and 50 μM CP, respectively, and processed at the indicated times. Western blot analyses were performed using monoclonal antibodies to p53 and caspase-2 as described in "Materials and Methods."

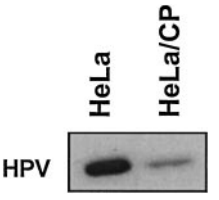

Fig. 2. Comparison of HPV level in HeLa and HeLa/CP cells. Western blot analysis was performed with total cellular extracts from HeLa and HeLa/CP cells using antibody raised against HPV E6 protein.

tion, whereas an inhibitor of PKC-δ prevented CP-induced caspase activation in HeLa cells (19, 20). Because PKC regulates sensitivity of HeLa cells to CP, and p53 is a substrate for PKC, we have examined whether an alteration in PKC influences p53 accumulation and cellular sensitivity/resistance to CP. Our results show that in parental HeLa cells, DNA damage is not sufficient to cause p53 accumulation, because PKC-mediated phosphorylation of p53 can lead to its degradation. A deregulation of PKCδ in CP-resistant HeLa cells may allow stabilization of p53 by DNA damage.

Materials and Methods

Materials. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide and CP were purchased from Sigma (St. Louis, MO). PDBu, Rottlerin, and Gö 6976 were purchased from LC Service Corporation (Woburn, MA), and MG132 was from Calbiochem (La Jolla, CA). Monoclonal antibody to p53 and HPV, and polyclonal antibody to PKCδ were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to caspase-2 was purchased from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated goat antimouse and donkey antirabbit antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA), and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL). Cisplatin was a generous gift from Bristol-Myers Squibb Co. (Wallingford, CT).

Cell Culture. Parental (HeLa) and CP-resistant (HeLa/CP) human cervical carcinoma cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. The cells were maintained in an incubator at 37°C with 95% air and 5% CO_2.

Immunoblot Analyses. Cells were treated with or without CP and PKC modulators. At the end of the incubation, cells were collected and washed twice with cold PBS, and lysed with mammalian protein extraction reagent buffer (Pierce) containing protease inhibitors and DTT. To extract nuclear proteins, cells were swelled in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor mixture] and lysed with NP40, and centrifuged for 1 min. Nuclear pellet was resuspended in buffer C [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 25% glycerol], vortexed vigorously and centrifuged at 16,000 $\times$ *g* for 20 min. The cytosolic fraction contained nuclear proteins. Equal amounts of protein were separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (24). Western blot analyses were performed, and the proteins were visualized using enhanced chemiluminescence reagents according to the manufacturer's protocol. Intensities of immunoreactive proteins were quantified by laser densitometry. A monoclonal antibody to caspase-2 (Transduction Laboratories) that did not alter during various treatment conditions was used as an internal control to account for any variability associated with the amount of protein loaded in each experiment during electrophoresis. We were unable to use actin as a loading control because high levels of actin in HeLa cells interfered with the linearity of the band intensities, especially because we had to load large amounts of protein because of low abundance of p53 in HeLa cells.

Assessment of Apoptosis by Flow Cytometric Analysis. Cells were treated with PKC modulators and CP as described in the text. At the end of the incubation, cells were harvested and washed with PBS. Nuclei were isolated and stained with propidium iodide as described before (25). Cell cycle analysis was performed using a flow cytometer (Coulter-Epics).

Results

Effect of Cisplatin on p53 Levels in HeLa and HeLa/CP Cells. Because p53 is induced in response to DNA damage, we examined the effect of CP on p53 content in parental HeLa cells and its CP-resistant variant, HeLa/CP cells. Because the level of CP resistance varied between 3- and 5-fold during the course of our experiments, we initially treated HeLa and HeLa/CP cells with 10 μM and 50 μM CP, respectively. As shown in Fig. 1, the basal p53 level was quite low in HeLa cells, and treatment with CP for 3 to 24 h had little effect on the p53 content. In contrast, HeLa/CP cells contained detected levels of p53, and CP caused a time-dependent increase in p53 in HeLa/CP cells (Fig. 1). We have used a monoclonal antibody of caspase-2 from Transduction Laboratories as a loading control because it was not altered by the treatment conditions in several independent experiments. The low level of p53 in HeLa/CP cells after a 24-h exposure to CP could be explained by loading differences. Because in HeLa cells, p53 is degraded via interaction with the E6 gene product of HPV, we compared the E6 level in HeLa and HeLa/CP cells using an antibody raised against HPV E6 protein. Fig. 2 shows that the level of HPV was

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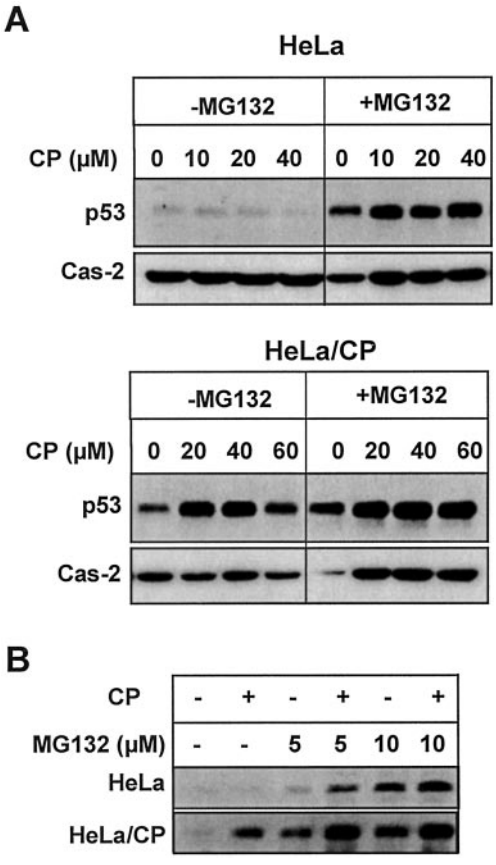

Fig. 3. Effect of MG132 on p53 levels in HeLa and HeLa/CP cells. *A,* HeLa and HeLa/CP cells were pretreated with or without 10 μM MG132 for 15 min and then with increasing concentrations of CP for 16 h. *B,* cells were pretreated with 5 μM or 10 μM MG132 and then treated with 20 μM CP for 16 h. Western blot analyses were performed as described in "Materials and Methods."

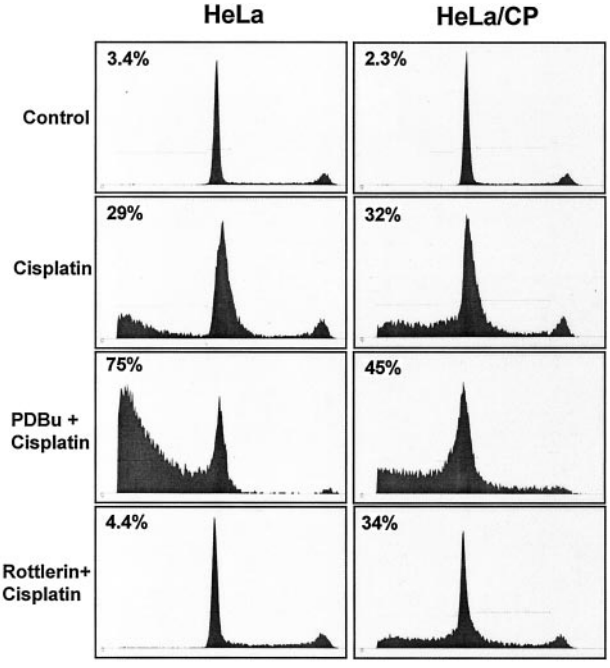

Fig. 4. Effects of PKC modulators on CP-induced apoptosis in HeLa and HeLa/CP cells. Cells were pretreated with or without 1 μM PDBu or 10 μM Rot and then treated with either 5 μM (HeLa) or 30 μM (HeLa/CP) CP for 22 h. Cells were then stained with propidium iodide and analyzed using a flow cytometer.

decreased in HeLa/CP cells compared with HeLa cells. This could explain why the constitutive p53 level was higher in CP-resistant HeLa cells compared with HeLa cells.

Treatment of HeLa cells with different concentrations of CP for 16 h also had little effect on the p53 content (Fig. 3). Because in HeLa cells, the E6 gene product of HPV interacts with p53, resulting in the rapid degradation of p53 through the ubiquitin-proteasome pathway (8), we examined the effect of the proteasome inhibitor MG132 on p53 content. As shown in Fig. 3*A,* a large amount of p53 could be detected in MG132-treated HeLa cells, suggesting that the rapid degradation of p53 by ubiquitin proteasome-mediated pathway prevents its accumulation in parental HeLa cells. Cisplatin caused an increase in p53 in HeLa/CP cells up to 40 μM. The ability of CP to induce p53 in HeLa/CP cells was attenuated at 60 μM concentrations, and it was prevented by the pretreatment of HeLa/CP cells with MG132. Thus, a lack of p53 accumulation in response to high concentrations of CP in HeLa/CP cells could also be explained by the proteasome-mediated degradation of p53. Fig. 3*B* shows that MG132 prevented degradation of p53 in a concentration-dependent manner in both HeLa and HeLa/CP cells. However, high concentrations of MG132 (20 μM) alone were toxic to these cells (data not shown).

Effect of PKC Modulators on Cisplatin-induced Apoptosis. We have shown previously that PKC activators enhance cellular sensitivity to CP (16–18). In Fig. 4, we have monitored apoptotic cell death by the appearance of hypodiploid peak in a flow cytometer. On the basis of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay, the concentrations of CP required to induce 50% cell death were 8.5 μM and 45 μM in HeLa and HeLa/CP cells, respectively. Because the IC_{50} values of the parental and CP-resistant HeLa cells differ by 5–6-fold, and because the effects of PKC activators were more pronounced at low concentrations of CP, we examined the effect of 5 μM and 30 μM CP in HeLa and HeLa/CP cells, respectively. In untreated HeLa cells, 3% of cells appeared in the sub-G_1 phase, and a 22 h treatment with 5 μM CP increased the apoptotic peak to 30%. Thirty μM of CP resulted in equivalent cell death (32%) in HeLa/CP cells. PKC activator PDBu alone had little effect on cell death (data not shown), but it increased apoptotic peak to 75% and 45% in HeLa and HeLa/CP cells, respectively, when used in conjunction with CP. We have also examined the effect of PKC inhibitors on CP-induced cell death. Rottlerin inhibits PKCδ with IC_{50} values of 3–6 μM with little effect on other PKC isozymes at $<$30 μM concentrations (26). Ten μM of Rot decreased fractions of cells undergoing apoptosis from 30% to $<$5% in HeLa cells, but it did not prevent cell death in HeLa/CP cells. Gö 6976 has been shown to inhibit cPKCα and -βI at nanomolar concentrations but had no effect on other PKC isozymes (27). We have found

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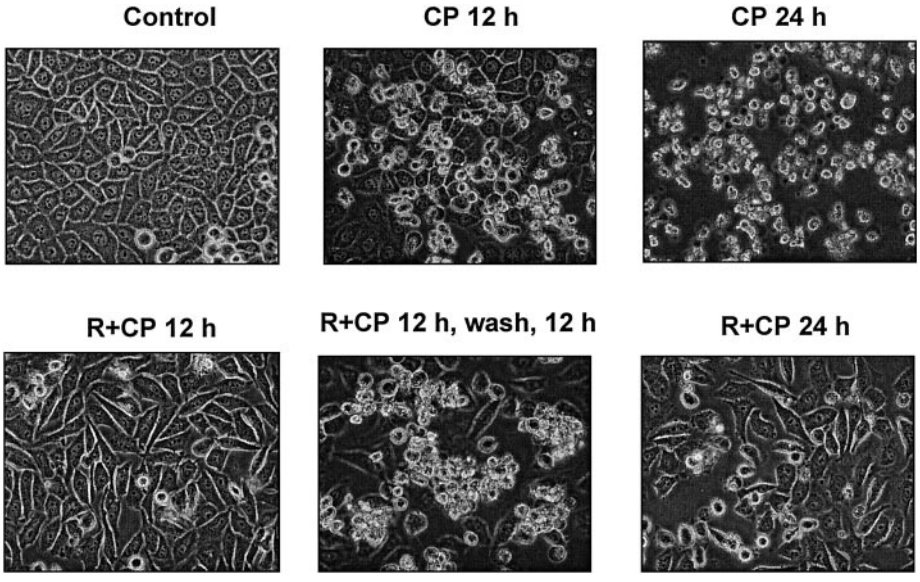

Fig. 5. Effect of Rot on CP-induced apoptotic morphology. HeLa cells were pretreated with (*bottom panels*) or without (*top panels*) 10 μM Rot for 30 min and then treated with or without 20 μM CP for 12 or 24 h. In one experiment, cells were washed after treatment with Rot and CP for 12 h and then incubated in the drug-free medium for 12 h. The cellular morphology was determined using a microscope.

that Gö 6976 by itself enhanced sub-G_1 cell population to 7–10%, and it caused 50–60% cell death in both HeLa and HeLa/CP cells (data not shown).

We have shown before that Rot was able to inhibit cell death if added together with or 1 h after CP addition, but the ability of Rot to prevent cell death decreased gradually when the time of addition of Rot was prolonged after CP treatment (20). Furthermore, Rot was unable to prevent cell death when added after CP was removed. Because DNA damage and caspase activation that leads to apoptotic cell death are coupled, and because Rot regulates caspase activation (19, 20), it is necessary to treat cells with Rot together with CP to inhibit cell death. Therefore, to examine whether Rot acts at a step before or subsequent to DNA damage, we pretreated HeLa cells with or without 10 μM Rot for 30 min and then treated with CP for 12 h or 24 h. As shown in Fig. 5, treatment of cells with CP resulted in the detachment of cells from the tissue culture dish, and the number of floating cells increased in a time-dependent fashion. Pretreatment of cells with Rot reversed the effect of CP. However, when cells were washed after treatment with Rot and CP for 12 h and then incubated in the drug-free medium, cells regained cellular morphology indicative of apoptosis. If the ability of Rot to prevent CP-induced cell death were because of its ability to inhibit CP uptake then the effect of Rot could not be reversed. These results suggest that Rot acts at a step subsequent to CP-induced DNA damage.

Effect of PKC Modulators on p53 Levels in HeLa and HeLa/CP Cells. Because p53 is a substrate for PKC, which regulates CP-induced cell death, we examined the effects of PKC modulators on p53 content in HeLa cells. Although Rot by itself had little effect on the p53 level, it caused a substantial increase in p53 when treated in combination with CP (Fig. 6*A*). On the basis of densitometric scanning of four

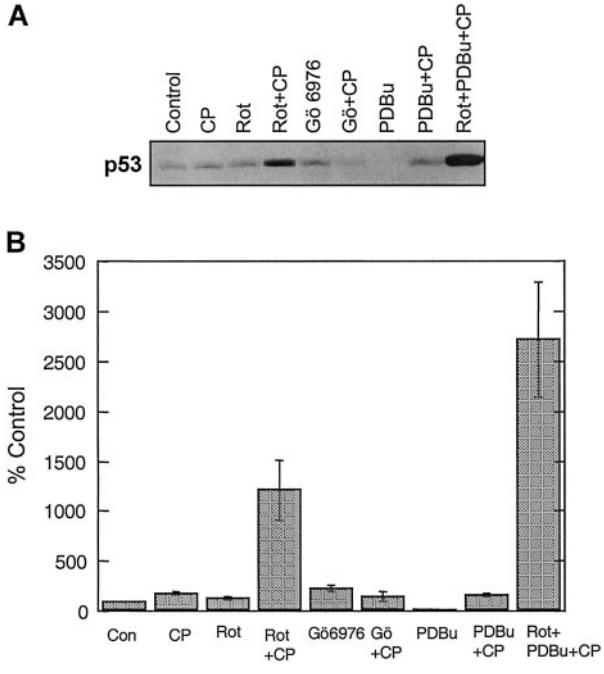

Fig. 6. Effect of PKC modulators on p53 content in HeLa cells. Cells were pretreated with or without 10 μM Rot, 5 μM Gö 6976, or 1 μM PDBu, and then treated with or without 20 μM CP for 16 h. *A,* Western blot analysis was performed using a monoclonal antibody to p53 as described in "Materials and Methods." *B,* the abundance of p53 was quantified by scanning immunoblots with a laser densitometer, and the values are the means of two to five individual experiments; *bars,* $\pm$SE.

independent experiments, Rot increased p53 level by 12-fold (Fig. 6*B*). To determine whether this effect was specific for novel PKC-δ, cells were treated with Gö 6976, an inhibitor of conventional PKCs. Gö 6976 by itself caused a slight in-

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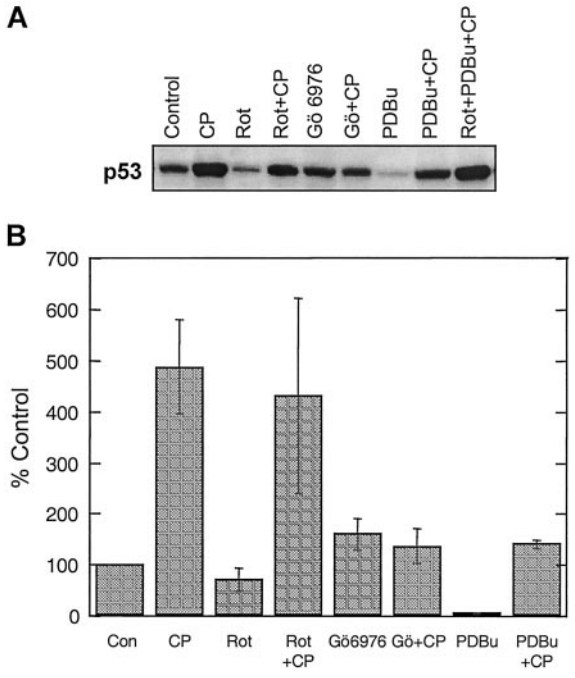

Fig. 7. Effect of PKC modulators on p53 levels in HeLa/CP cells. Cells were pretreated with or without 10 μM Rot, 5 μM Gö 6976, or 1 μM PDBu, and then treated with or without 50 μM CP for 16 h. *A,* Western blot analysis was performed with total cellular extracts using a monoclonal antibody to p53 as described in "Materials and Methods." *B,* the abundance of p53 was quantified by scanning immunoblots with a laser densitometer, and the values are the means of two to five individual experiments; *bars,* ±SE.

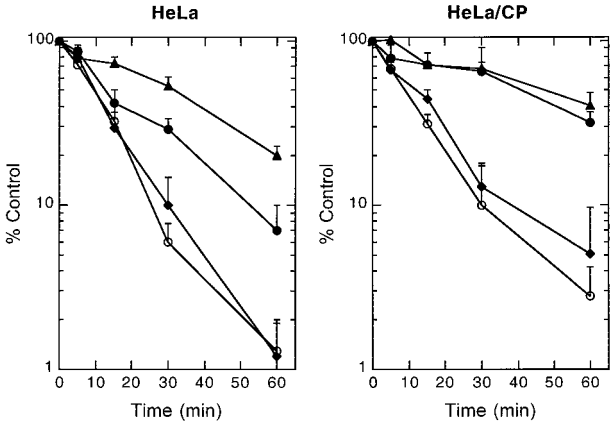

Fig. 8. Effect of inhibition of protein synthesis on p53 levels in HeLa and HeLa/CP cells. HeLa and HeLa/CP cells were pretreated with or without 10 μM Rot for 30 min and then treated with or without CP for 16 h. Cells were then treated with 10 μg/ml cycloheximide, and nuclear extractions were performed at the indicated times as described in "Materials and Methods." Densitometric scanning was performed on Western blots from three to five separate experiments. Values were plotted as a percentage of the control. ○, control; ●, CP; ♦, Rot; ▲, Rot+CP; *bars,* ±SE.

crease in p53 level but it failed to increase p53 when treated in conjunction with CP (Fig. 6*B*). The PKC activator PDBu resulted in additional decrease in p53 levels. These results suggest that activation of PKC results in degradation of p53 and inhibition of novel PKCδ rather than conventional PKCs was responsible for the increase in p53 in response to CP. When cells were treated with combination of PDBu and CP, the decrease in p53 by PDBu was attenuated. Furthermore, pretreatment of cells with Rot counteracted the effect of PDBu on the decrease in p53. In fact, combination of PDBu, Rot, and CP was even more effective than Rot and CP to enhance p53 level.

We also examined the effects of PKC modulators on p53 levels in HeLa/CP cells (Fig. 7). Treatment of HeLa/CP cells with CP resulted in a 5-fold increase in p53 content (Fig. 7*B*). Rottlerin had little effect on the accumulation of p53. Gö 6976 by itself resulted in a slight increase in p53 level, but it caused a decrease in CP-induced p53 accumulation. The PKC activator PDBu not only decreased the constitutive level of p53 in HeLa/CP cells but it also attenuated the induction of p53 by CP (Fig. 7*B*). Thus, CP caused an accumulation of p53 in HeLa/CP cells, whereas activation of PKC resulted in the disappearance of p53.

Effect of Rottlerin and Cisplatin on the Stabilization of p53 in HeLa and HeLa/CP Cells. Because treatment with a combination of Rot and CP caused a significant increase in p53 levels in HeLa cells, we examined the effect of Rot and CP on the stabilization of p53 (Fig. 8). HeLa and HeLa/CP cells were pretreated with or without Rot and then treated either with or without CP. After a 16-h incubation, *de novo* protein synthesis was inhibited by treating cells with 10 μg/ml cycloheximide, and cells were incubated for the indicated time periods. In untreated HeLa cells, p53 was rapidly degraded, and Rot had little effect on the stabilization of p53. The half-lives of untreated and Rot-treated HeLa cells were estimated to be $<$10 min. p53 was also rapidly degraded in HeLa cells treated with CP with a half-life of 12 min. However, a small amount of p53 could be detected even at 60 min in CP-treated cells. A combined treatment with CP and Rot increased the half-life of p53 to $\sim$30 min. p53 was also rapidly degraded in HeLa/CP cells with a half-life of $\sim$10 min, and treatment with Rot increased the half-life slightly to 12 min. In HeLa/CP cells, CP alone caused a substantial increase in the half-life of p53 to 40 min, and combination of Rot and CP had little additional effect on the half-life of p53. Thus, whereas both Rot and CP were required to stabilize p53 in parental HeLa cells, CP alone caused considerable stabilization of p53 in the CP-resistant HeLa cells.

Effect of Rottlerin on Cisplatin-induced Proteolytic Activation of PKCδ in HeLa and HeLa/CP Cells. Because PKC regulates p53 stabilization and CP induces proteolytic activation of PKCδ, we compared the ability of CP to induce proteolytic activation of PKCδ in CP-sensitive and -resistant HeLa/CP cells. As shown in Fig. 9, treatment of HeLa cells with 10 μM of CP caused a decrease in full-length PKCδ with a concomitant increase in the catalytic fragment of PKCδ, and proteolytic cleavage of PKCδ was increased additionally by 20 μM of CP. Although 20 μM of CP had little effect on the proteolytic cleavage of PKCδ in HeLa/CP cells, the PKCδ catalytic fragment generated by 40 μM of CP in HeLa/CP cells was equivalent to that generated by 10 μM of CP in HeLa cells, once corrected for loading differences based on the level of PKCα. However, whereas pretreatment of HeLa

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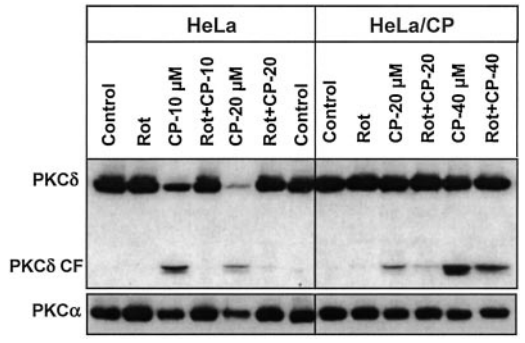

Fig. 9. Comparison of the effect of CP and Rot on the proteolytic activation of PKCδ in HeLa and HeLa/CP cells. Cells were pretreated with or without 10 μM Rot and then treated with indicated concentrations of CP for 16 h. Western blot analysis was performed with a polyclonal antibody to PKCδ or monoclonal antibody to PKCα.

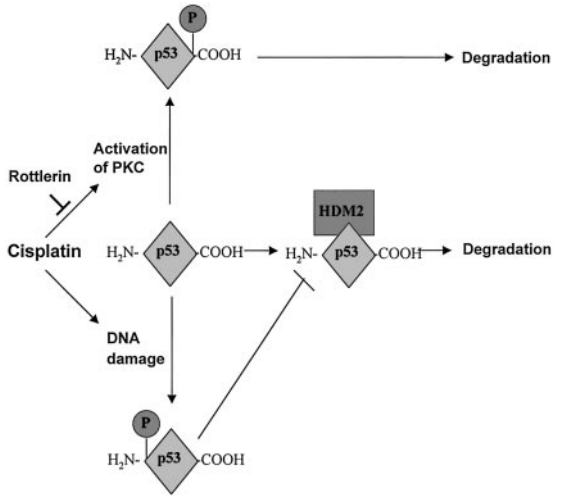

Fig. 10. Regulation of p53 stability by DNA damage and PKC.

cells with Rot blocked proteolytic activation of PKCδ induced by CP, Rot was unable to prevent proteolytic cleavage of PKCδ in HeLa/CP cells. Thus, the ability of Rot to prevent proteolytic cleavage of PKCδ was compromised in HeLa/CP cells.

Discussion

The p53 tumor suppressor protein is at the core of the DNA damage-induced signaling pathway and plays a critical role in determining the cellular outcome of DNA damage. Post-translational modification of p53 by phosphorylation is the major mechanism of p53 regulation. The results of our present study demonstrated that the PKC signal transduction pathway plays an important role in regulating DNA damage-induced stabilization of p53. Whereas inhibition of PKCδ as well as CP-induced DNA damage were necessary for the stabilization of p53 in HeLa cells, CP alone was sufficient to stabilize p53 in CP-resistant HeLa cells. Furthermore, an increase in CP-induced p53 accumulation was associated with a decrease in apoptosis, suggesting that CP induced a p53-independent apoptosis in HeLa cells.

Normally p53 is rapidly degraded because of interaction with the oncoprotein MDM2 at its NH_2 terminus, which targets its degradation via ubiquitin-proteasome mediated pathway (7, 9–11). In response to DNA damage, p53 becomes phosphorylated at its NH_2 terminus, thereby inhibiting MDM2 from binding p53, thus preventing degradation of p53 (9–13). In HeLa cells, CP-induced DNA damage was not sufficient to accumulate p53. Treatment with the proteasome inhibitor MG132 resulted in an increase in p53 level. This is consistent with the report that in HPV-positive HeLa cells, p53 gets degraded via ubiquitin-protein ligase and, therefore, p53 is not stabilized in response to DNA damage (28). We have found that the HPV E6 level was attenuated in HeLa/CP cells compared with HeLa cells. Furthermore, treatment of HeLa cells with CP caused a decrease in HPV level (data not shown). It is conceivable that during selection with CP, HPV level was decreased in HeLa/CP cells, and this may explain why basal p53 level was higher in HeLa/CP cells compared with HeLa cells. However, this does not explain why CP failed to induce p53 content in HeLa cells.

PKC is also able to regulate p53 by phosphorylation at the COOH terminus of p53. PKC-dependent phosphorylation at the COOH terminus of p53 also leads to the ubiquitination and subsequent degradation of p53 (14, 15). It has been shown that a nonspecific PKC inhibitor H7 or a PKC-specific inhibitor bisindolylmaleimide increased p53 to a very high level in mouse and human cell lines (15). In HeLa cells, neither DNA damage nor PKC inhibitors were able to cause accumulation of p53. However, a combination of PKCδ inhibitor Rot and CP caused a dramatic increase in p53 content. Furthermore, combination of PDBu, Rot, and CP was even more effective than Rot and CP to enhance p53 level. Because prolonged exposure of HeLa cells to PDBu during treatment with CP for 16 h induces down-regulation of PKCδ, PDBu may cooperate with the PKCδ inhibitor to increase p53 content in the presence of CP. By contrast, CP alone caused an increase in p53 in HeLa/CP cells, and Rot was unable to additionally increase p53 level after treatment with CP. In both HeLa and HeLa/CP cells, the cPKC inhibitor Gö 6976 by itself caused a modest increase in p53. Because Gö 6976 was somewhat toxic to HeLa cells and it induced cell death by itself, its effect on p53 could be because of a stress response. Both PDBu and Gö 6976, which enhanced CP-induced cell death, resulted in a decrease in p53 accumulation. Thus, in HeLa cells, CP-induced apoptosis appears to be p53-independent. The mechanisms of CP resistance are multifactorial. These include drug uptake, DNA repair, binding of CP to cellular thiols, and inhibition of apoptosis. These mechanisms are not mutually exclusive ,and more than one mechanism is usually operative even in the same cell type. We have shown that in HeLa/CP cells Rot was unable to prevent cell death by CP. It also failed to prevent proteolytic cleavage of PKCδ and did not enhance accumulation of p53 by CP. This is consistent with our observation that the regulation of PKC is compromised in CP-resistant cells (29, 30).

On the basis of the existing literatures (14, 15) and findings from the present study, we propose a hypothetical model (Fig. 10) to account for differences observed in HeLa and HeLa/CP

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cells. We propose that phosphorylation of NH_2-terminal sites as well as dephosphorylation of the PKC site at the COOH-terminal domain were necessary for the stabilization of p53 in HeLa cells. PDBu caused a decrease in p53 in both HeLa and HeLa/CP cells, suggesting that phosphorylation of p53 by PKC activator led to its degradation. Because treatment with CP results in the proteolytic activation of PKCδ, CP-induced DNA damage not only causes NH_2-terminal phosphorylation but it may also enhance phosphorylation of p53 at the COOH-terminal domain. We have shown that Rot prevents CP-induced proteolytic activation of PKCδ. Because Rot acts subsequent to DNA damage, it would not interfere with DNA damage-induced NH_2-terminal phosphorylation of p53. Therefore, the combination of Rot and CP would induce phosphorylation at the NH_2-terminal site as well as dephosphorylation at the COOH-terminal PKC site, thereby resulting in the stabilization of p53. In HeLa/CP cells, Rot was unable to prevent proteolytic activation of PKCδ. It also failed to enhance CP-induced accumulation of p53. We also observed that the ability of PKC activators to down-regulate PKC was attenuated in HeLa/CP cells compared with HeLa cells,[4] suggesting that the PKC signal transduction pathway is deregulated in HeLa/CP cells. Thus, CP alone was sufficient to cause stabilization of p53. Stabilization of the p53 protein, and its subsequent accumulation, is important in regulating p53 function. Our results suggest that an increase in p53 may not be associated with enhanced cell death.

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