

# A New Role of Protein Phosphatase 2A in Adenoviral E1A Protein-Mediated Sensitization to Anticancer Drug-Induced Apoptosis in Human Breast Cancer Cells

Yong Liao and Mien-Chie Hung

Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

## Abstract

The adenoviral type 5 E1A protein has been shown to induce sensitization to different categories of anticancer drug-induced apoptosis, partly by down-regulation of the activity of a critical oncogenic kinase Akt in both normal fibroblasts and epithelial breast cancer cells. Currently, the adenoviral E1A gene is being tested as an antitumor gene in multiple clinical trials. However, molecular mechanisms underlying E1A-mediated chemosensitization and down-regulation of Akt activity are still not completely defined. Here, we show that E1A by up-regulation of the catalytic subunit of protein phosphatase 2A [PP2A (PP2A/C)] enhanced the activity of PP2A, which results in repression of Akt activation in E1A-expressing cells. In addition, activation of PP2A/C is required for E1A-mediated sensitization to drug-induced apoptosis, because blocking PP2A/C expression using a specific small interfering RNA against PP2A/C reduced drug sensitivity in E1A-expressing cells. Deletion mutation of the conserved domain of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, also abolished the ability of E1A to up-regulate PP2A/C. Thus, the up-regulation of PP2A may represent a novel mechanism for E1A-mediated sensitization to anticancer drug-induced apoptosis.

## Introduction

Reversible phosphorylation of proteins by protein kinases and phosphatases is a key regulatory mechanism in the control of multiple cellular processes, ranging from cell proliferation and survival to cell death. Many oncogenes identified today are protein kinases; because their kinase activities are finely regulated by respective protein phosphatases, it is important to understand how the respective protein phosphatases are involved in the regulation of these cellular processes (1). Thus far, alterations or mutations of very few phosphatases have been implicated in the development of tumors, and only the dual-specific protein phosphatase PTEN exhibits many characteristics of a typical tumor suppressor (2, 3). A tumor-suppressive function of protein phosphatase 2A (PP2A) has been proposed, because a deletion mutation of the regulatory subunit of PP2A was found in primary human breast, colon, and lung tumors and melanoma (2) and inactivation of PP2A by the small t antigen of the DNA tumor virus SV40 was also found to be involved in viral-induced cell transformation (4). The core enzyme of PP2A is a dimer, consisting of a catalytic subunit (PP2A/C) and a regulatory or structural A subunit (PP2A/A). A third regulatory B subunit (PP2A/B), which determines substrate specific-

ity, can be associated with this core structure (1, 2). Recent evidence indicates that PP2A forms stable complexes with protein kinase signaling molecules, indicating that it plays a central, regulatory role in signal transduction mediated by reversible protein phosphorylation (1). Although the role of PP2A in the regulation of apoptosis is not clear, results from a gene knockout study of PP2A/C imply that it may play a critical role in regulation of apoptotic signaling (5). In supporting of this notion, several groups reported that PP2A through the dephosphorylation of a key oncogenic survival factor Akt participated in the regulation of apoptosis induced by ceramide, mistletoe lectin, and 4-hydroxynonenal, an aldehyde product of membrane lipid peroxidation (6–8).

Recently, we and other groups have shown that repression of Akt activation by adenoviral E1A contributed to E1A-mediated sensitization to anticancer drug-induced apoptosis in both normal fibroblast and epithelial breast cancer cells (9–11). However, the mechanism involved in E1A-mediated repression of Akt activity is still not known. Here, we show that the PP2A phosphatase activity is enhanced in E1A-expressing cells through E1A-mediated up-regulation of PP2A/C expression, which results in repression of Akt activation. We demonstrated that PP2A is involved in regulation of apoptosis and that activation of PP2A/C is also required for E1A-mediated sensitization to anticancer drug-induced apoptosis in E1A-expressing breast cancer cells.

## Materials and Methods

**Cell Lines.** The stable E1A-expressing cell lines and domain deletion mutants were established as described previously (10).

**Preparation of Cell Lysates, Western Blot Analysis, and Antibodies.** Preparation of cell lysates and Western blot analysis were performed according to standard protocols as described previously (10). Rabbit antihuman PP2A/A and PP2A/C were purchased from CalBiochem (La Jolla, CA). Information of other antibodies used was described previously (10).

**Serine/Threonine Phosphatase Assay.** A nonradioactive serine/threonine phosphatase assay system was purchased from Promega Corporation (Madison, WI). PP2A phosphatase activity was measured according to the manufacturer's protocol.

**Immunoprecipitation, Protein Phosphatase 2A Treatment, and Akt Dephosphorylation Assay.** To measure endogenous Akt dephosphorylation by exogenous purified recombinant human PP2A (Upstate Biotechnology, Lake Placid, NY), cells were stimulated using 10  $\mu$ mol/L insulin for 15 minutes before harvesting. To measure exogenous Akt dephosphorylation, Hemagglutinin-tagged Akt was transiently transfected into 293T cells. Cells were lysed and immunoprecipitated with anti-Akt or anti-HA antibodies. Aliquots of the Akt immunoprecipitates were incubated with various doses of recombinant human PP2A at 30°C for 30 minutes, after which the reaction was terminated through the addition of 6 $\times$  SDS loading buffer and resolved using 10% SDS-PAGE. Phosphorylated and total Akt and HA-tag were measured using anti-phospho-Akt (Thr-308-p) and total Akt and anti-HA antibody, respectively.

**Synthesis and Application of Small Interfering RNA.** Double-stranded small interfering RNAs were produced *in vitro* using chemically synthesized

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**Requests for reprints:** Mien-Chie Hung, Department of Molecular & Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. Phone: 713-792-3668; Fax: 713-794-0209; E-mail: mhung@mdanderson.org.

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DNA oligonucleotide templates (Sigma, St. Louis, MO) and the T7-MEGA-shortsript kit (Ambion, Inc., Austin, TX) according to the T7 small interfering RNA protocol described by Paddison *et al.* (12). The specific primer sequences for PP2A/C are as follows: A, 5'-CCG AGT CCC AGG TCA AGA G CC TAT AGT GAG TCG TAT TAC-3'; and B, 5'-GAG GCT CTT GAC CTG GGA C CC TAT AGT GAG TCG TAT TAC-3'. The nonspecific scrambled control primer sequences are as follows: A, 5'-ATG GAG AGC AGG TCA AAC T CC TAT AGT GAG TCG TAT TAC-3'; B, 5'-TTG GAG TTT GAC CTG CTC T CC TAT AGT GAG TCG TAT TAC-3'.

**Statistical Analysis.** Statistical analysis was performed with a two-tailed Student's *t* test, and  $P < 0.05$  was considered statistically significant.

## Results and Discussion

**Protein Phosphatase 2A Activity Is Enhanced, Which Is Correlated with Elevated Expression of the Catalytic Subunit of Protein Phosphatase 2A in Stable E1A-Expressing Cells.** Phosphorylation of protein kinases are tightly regulated by related protein phosphatases, and two phosphatases, PTEN and PP2A, have been shown to repress Akt activation through dephosphorylation (3, 6–8). To identify whether protein phosphatases were involved in E1A-mediated down-regulation of Akt activation, we measured the alteration of protein phosphatases, such as PTEN and PP2A, in stable E1A-expressing cells *versus* that in vector control cells. We did not detect any change in PTEN expression in stable E1A-expressing cells *versus* control cells (data not show). Also, there was no change in the expression level of the PP2A regulatory A subunit PP2A/A, however, we did detect elevated expression of the catalytic subunit of PP2A

(PP2A/C) in multiple stable E1A-expressing cells (Fig. 1A). Thus, we further tested whether PP2A activity was increased in the E1A-expressing cells by using a specific PP2A phosphatase assay. We observed that the PP2A activity was enhanced in E1A-expressing MDA-MB-231 cells (231-E1A) in a dose (protein and substrate concentration)-dependent manner compared with that of the vector control cells (231-Vect; both  $P < 0.01$ ; Fig. 1B). The above results suggest that E1A, by up-regulating PP2A/C expression, enhances the activity of PP2A.

### PP2A Is Involved in the Regulation of Akt and p38 Activities.

Next, we asked whether enhanced PP2A activity in E1A-expressing stable cells contributed to E1A-mediated repression of Akt activation. First, we verified whether Akt could be dephosphorylated by recombinant human PP2A, which contains both the catalytic and the regulatory A subunits. Dephosphorylation of endogenous Akt occurred in a recombinant human PP2A dose-dependent manner (Fig. 1C). Similar to the dephosphorylation of endogenous Akt, the HA-tagged, exogenous Akt was also dephosphorylated by recombinant human PP2A in a dose-dependent manner *in vitro* (data not show). To test whether dephosphorylation of Akt is dependent on PP2A activity, we used the specific PP2A inhibitor okadaic acid to block PP2A activity and measured Akt phosphorylation in the presence of recombinant human PP2A. We found that dephosphorylation of both endogenous and exogenous Akt was completely abolished in the presence of 1 nM PP2A inhibitor okadaic acid (Fig. 1C; data not show). This result suggests that

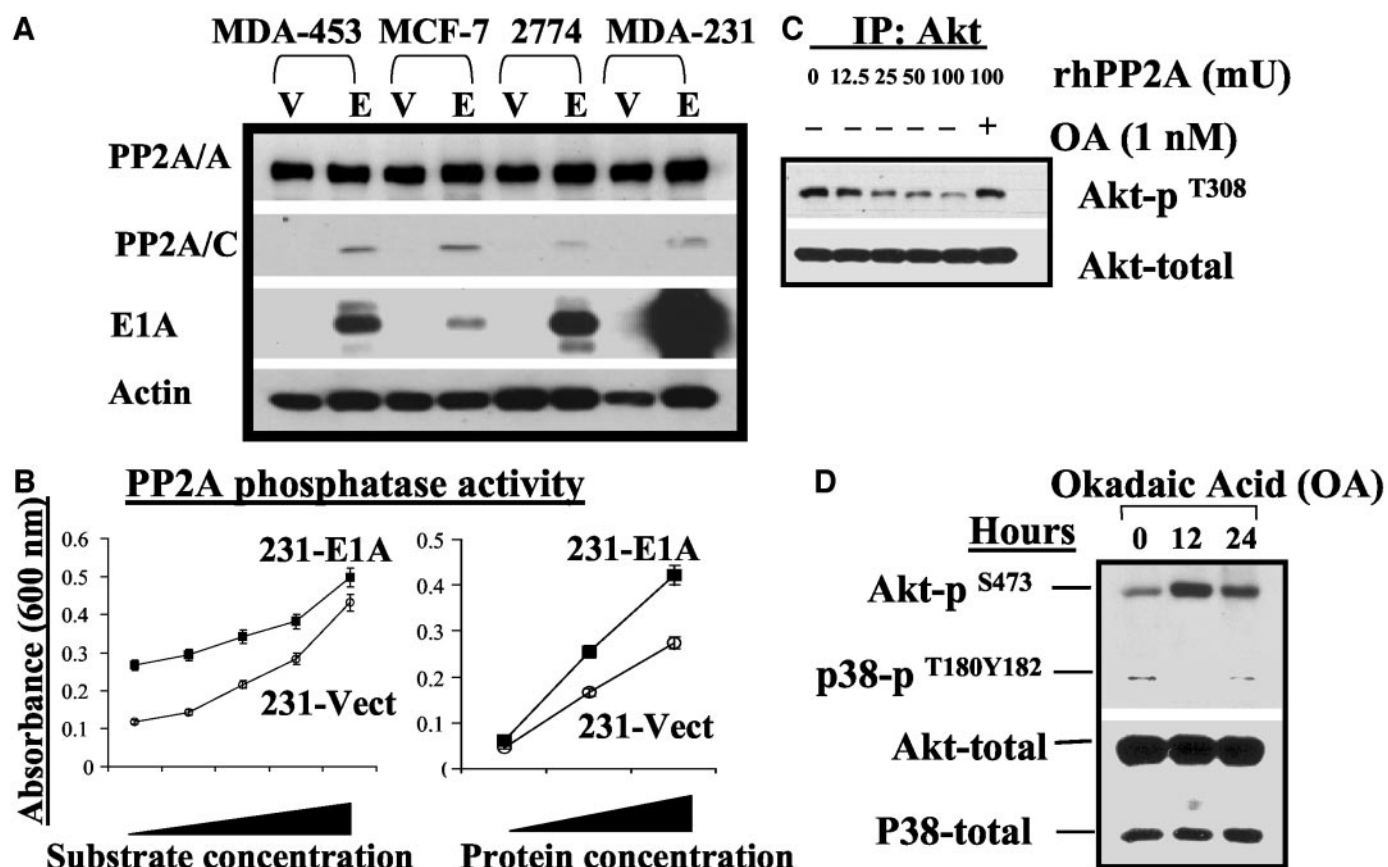


Fig. 1. PP2A activity is enhanced in stable E1A-expressing cells through up-regulation of PP2A/C. **A**, Protein expression of PP2A/A, PP2A/C, E1A, and actin in stable E1A-expressing cells (E) and corresponding vector controls (V). **B**, PP2A activity was measured using a protein phosphatase assay kit. The substrate phospho-peptide concentrations were 20, 40, 100, 200, and 400  $\mu\text{mol/L}$ , respectively. Protein concentrations include 0.1, 1.0, and 10  $\mu\text{g}$ , respectively. V, 231-Vector. E, 231-E1A. Results shown here are from free independent experiments. **C**, Dephosphorylation of Akt by purified hPP2A (1 milliunit =  $1 \times 10^{-3}$  units) and inhibition of by okadaic acid (1 nmol/L) *in vitro*. Aliquots of immunoprecipitated endogenous Akt were incubated with purified human PP2A enzyme (rhPP2A); relative phosphatase activities were measured by anti-phospho-Akt (T308) antibody. Total Akt was used as a loading control. **D**, Blocking PP2A activity by exposing 231-E1A cells to okadaic acid (10 nmol/L) increased Akt phosphorylation and inhibited p38 phosphorylation.

dephosphorylation of Akt is mediated by PP2A and dependent on PP2A phosphatase activity *in vitro*.

To test whether dephosphorylation of Akt also occurs in cells *in vivo*, stable E1A-expressing 231-E1A cells were treated with okadaic acid, and the Akt phosphorylation level was monitored for 24 hours. In the presence of okadaic acid, the Akt phosphorylation level was significantly increased at 12 hours, and the increment was subsequently reduced at 24 hours, presumably due to the limited half-life of okadaic acid (Fig. 1D). Previously, we and other groups have shown that activation of Akt results in inactivation of p38 (10, 13), therefore we also measured p38 phosphorylation before and after exposure with okadaic acid. Inconsistent with the previous studies, phosphorylation of p38 was detected before exposure to okadaic acid (0 hours) and was repressed at 12 hours when Akt phosphorylation was increased. Additionally, p38 phosphorylation was recovered at 24 hours when accumulation of Akt phosphorylation was reduced (Fig. 1D). Both

total Akt and total p38 protein levels had no change throughout exposure with okadaic acid (Fig. 1D). This result indicates that both Akt and p38 phosphorylation can also be regulated by PP2A *in vivo*.

**Up-regulation of Protein Phosphatase 2A/C Is Required for E1A-Mediated Chemosensitization.** It has been reported that E1A could sensitize anticancer drug-induced apoptosis through down-regulation of Akt activation (9, 10); we asked whether E1A-induced up-regulation of PP2A/C might play a role in E1A-mediated chemosensitization. To test this, we measured PP2A/C expression during anticancer drug-induced apoptosis in both 231-Vect cells and 231-E1A cells. We used poly(ADP-ribose) polymerase (PARP) cleavage as a marker of apoptosis and Bcl-2 phosphorylation as an indication of the pharmaceutical effect of the antimicrotubule drug paclitaxel (14). The protein level of PP2A/C was further increased and was correlated with reduced Akt phosphorylation, increased p38 phosphorylation and Bcl-2 phosphorylation, and increased PARP cleavage after exposure

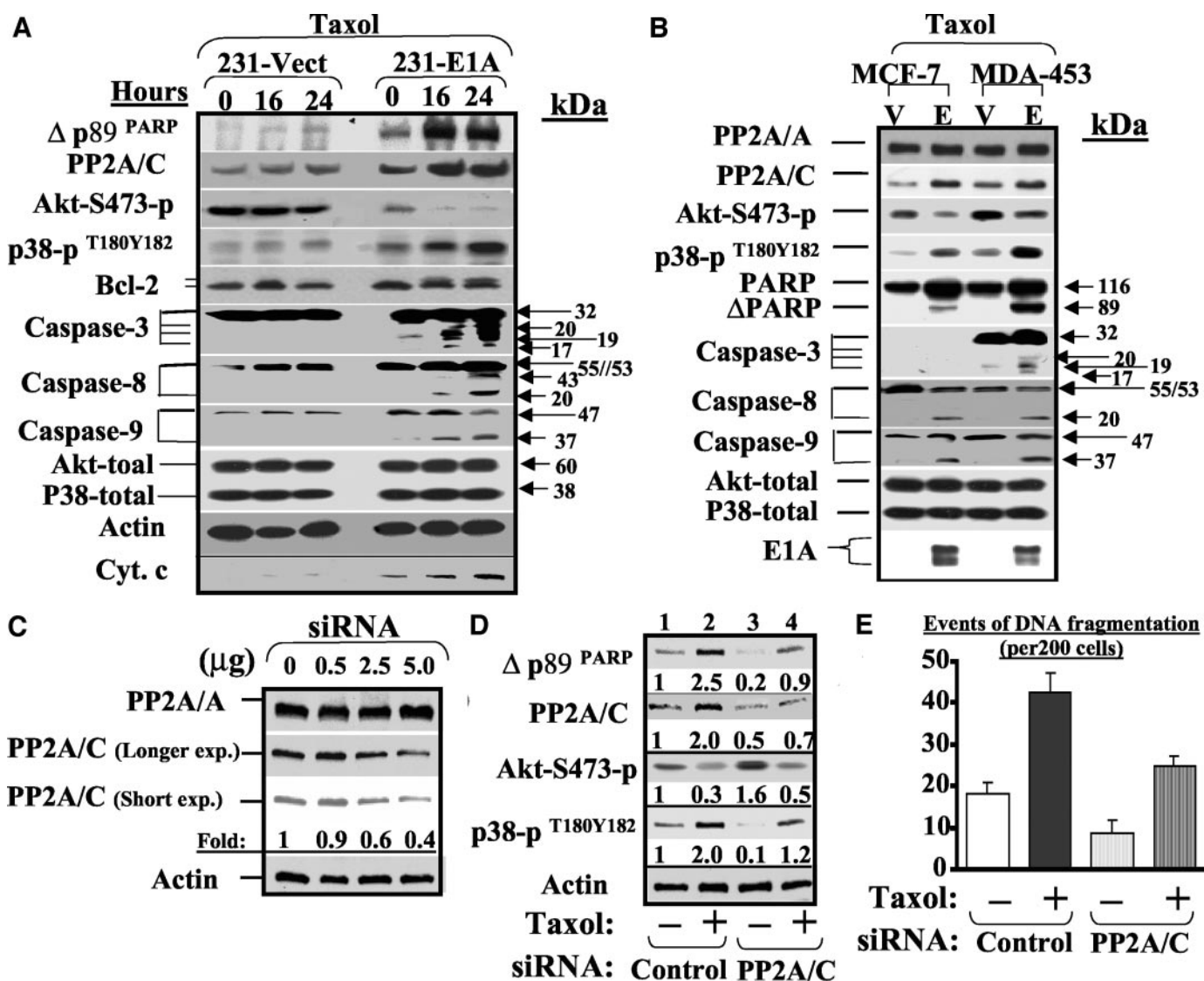


Fig. 2. Up-regulation of PP2A/C is required for E1A-mediated chemosensitization. **A**, PARP cleavage and PP2A/C activation in 231-Vect and 231-E1A cells after treatment with paclitaxel. *Cyt. c*, cytochrome *c*. **B**, Western blot analysis of PP2A and the catalytic subunit PP2A/C in Vect (V)- or E1A (E)-transfected MCF-7 and MDA-MB-453 cells after exposure to 0.01 and 1.0  $\mu\text{mol/L}$  paclitaxel, respectively. **C**, a dose-finding test of specific PP2A/C small interfering RNA on PP2A/C expression in 231-E1A cells. Relative intensity of PP2A/C was shown in the bottom. **D**, Cells were transfected with small interfering RNA using JetSI cationic transfection reagent (Obiogene, Inc., Carlsbad, CA) for 16 hours and replaced with fresh medium before addition of 0.01  $\mu\text{mol/L}$  paclitaxel and were incubated for another 24 hours before harvesting. Relative intensities of PARP and PP2A/C in specific small interfering RNA (PP2A/C) and nonspecific control small interfering RNA (Control) protected cells were shown in the bottom of each band. **E**, For nuclear fragmentation analysis, cells were grown in Lab-Tek Chamber Slides (Nunc, Inc., Naperville, IL) and were treated by the same procedure as described above. Cells were then washed with PBS twice, fixed with 70% alcohol, and stained with Hoechst 33342 (0.5  $\mu\text{g/mL}$ ; Sigma). Events of apoptotic nuclei were counted under a fluorescence microscope, and the mean values in every 200 cells in each field were plotted.

to paclitaxel in the 231-E1A cells, suggesting that increased PP2A/C is correlated with drug-induced apoptosis in E1A-expressing cells (Fig. 2A). In addition, to test which apoptotic pathway is involved in E1A-mediated sensitization to paclitaxel-induced apoptosis, we measured the expression of caspase-3, -8, -9, and cytosolic fraction of cytochrome *c* (15). We observed that paclitaxel-induced PP2A/C expression and PARP cleavage in 231-E1A cells correlated with cytochrome *c* release and activation of procaspase-8, -9, and -3. This result suggests that both intrinsic (represented by cytochrome *c* release and caspase-9 cleavage) and extrinsic (represented by activation of procaspase-8) apoptotic pathways may be involved in E1A-mediated sensitization to paclitaxel-induced apoptosis (15). PARP cleavage and increased PP2A/C was also observed in the 231-Vect cells after treatment with paclitaxel, but to a much lesser extent, it implied that PP2A/C may be required for drug-induced apoptosis in the absence of E1A.

To test whether the above observation can also be applied to E1A-mediated chemosensitization in other cell lines, additional two pairs of E1A-expressing stable cell lines and the vector DNA-transfected controls were tested. We observed notably higher levels of PP2A/C, reduced Akt phosphorylation, enhanced p38 phosphorylation, and cleaved PARP fragment in E1A-expressing cells after exposure to paclitaxel compared with that of the corresponding vector controls. Again, enhanced expression of PP2A/C and cleaved PARP fragment in E1A-expressing cells after exposure to paclitaxel correlated with the activation of procaspase-3, -8, and -9 (Fig. 2B), whereas the expression level of the regulatory subunit of PP2A/A was not significantly altered in E1A-expressing cells *versus* control cells (Fig. 2B). These data suggest that elevated expression of PP2A/C in E1A-expressing cells is involved in E1A-mediated sensitization to drug-induced apoptosis.

To further test whether up-regulation of PP2A/C expression by E1A is required for E1A-mediated sensitization to apoptosis, we used a double-stranded small interfering RNA against PP2A/C as a tool to knockdown PP2A/C expression in E1A-expressing cells. First, we did a dose escalation study of small interfering RNA on PP2A/C protein expression. We found 5  $\mu\text{g}$  of small interfering RNA were sufficient to repress PP2A/C expression ( $\sim 60\%$  reduction in PP2A/C protein expression) in 231-E1A cells (Fig. 2C). Second, we tested whether repression of PP2A/C expression would also inhibit drug-induced apoptosis in E1A-expressing cells. We exposed 231-E1A cells to either a specific small interfering RNA against PP2A/C or a scrambled, nonspecific control small interfering RNA in the presence or absence of paclitaxel for 24 hours. Third, we measured the expression levels of cleaved PARP and PP2A/C and counted events of nuclear fragmentation under microscopy as an alternative measure for the occurrence of apoptotic cells. We detected a 2-fold increase in the expression level of PP2A/C and 2.5-fold increase of cleaved PARP in the presence of paclitaxel compared with cells treated with control small interfering RNA alone without paclitaxel (Fig. 2D, Lanes 1 *versus* 2). However, when compared with cells in the presence of control small interfering RNA, cells treated with PP2A/C-specific small interfering RNA had reduced both PP2A/C expression and cleaved PARP (0.5-fold of PP2A/C and 0.2-fold cleaved PARP proteins; Fig. 2D, Lanes 1 *versus* 3). In the presence of both PP2A/C-specific small interfering RNA and paclitaxel, the increment of PP2A/C expression is minimal (0.7-fold that of control small interfering RNA alone) and the cleaved PARP protein is comparable with control small interfering RNA alone (0.9-fold) in the absence of paclitaxel (Fig. 2D, Lanes 1 *versus* 4). Inconsistent with the above results, we also observed that when PP2A/C expression was blocked by specific small interfering RNA, Akt phosphorylation was elevated, whereas p38 phosphorylation was reduced (Fig. 2D). Corresponding

with the expression of PP2A/C and cleaved PARP proteins, the rate of DNA fragmentation in control small interfering RNA-treated cells is about 9% (18 of 200) in the absence of paclitaxel and 21% (42 of 200) in the presence of paclitaxel, whereas in PP2A/C-specific small interfering RNA-treated cells, the rate of DNA fragmentation is about 4% (8 of 200) in the absence of paclitaxel and 12% (24 of 200) in the presence of drug (Fig. 2E).

Taken together, the above results suggest that up-regulation of PP2A/C is required for E1A-mediated sensitization to drug-induced apoptosis.

**A Protein Phosphatase 2A Phosphatase Activity Is Also Involved in Apoptosis Induced by Different Apoptotic Stimuli, Such as Tumor Necrosis Factor- $\alpha$ .** The above results established that by regulation of the Akt activation, PP2A played a role in adenoviral E1A-mediated sensitization to anticancer drug-induced apoptosis by repressing Akt activation and up-regulation of p38 activation. In the previous report, we have shown that repression of Akt activation and up-regulation of p38 activation contributed to different apoptotic stimuli-induced apoptosis, such as exposure to tumor necrosis factor (TNF)- $\alpha$  (10). To further test whether PP2A plays a general role in the regulation of apoptosis by different apoptotic stimuli in the absence of E1A, we treated MDA-MB-231 cells with TNF- $\alpha$  at a dose that could induce apoptosis (10). PP2A phosphatase activity was measured after treatment. As experimental controls, we also treated MDA-MB-231 cells with insulin-like growth factor-1, which is known to transiently stimulate the phosphatidylinositol 3'-kinase-Akt pathway, and MEK inhibitor PD58098, which is not supposed to affect Akt or p38 phosphorylation. We again used the cleaved PARP fragment (p89PARP) as a marker for apoptosis. As expected, we detected cleaved PARP in MDA-MB-231 cells after treatment with TNF- $\alpha$  for 24 hours, correspondingly, we detected increased p38 phosphorylation and reduced Akt phosphorylation. Interestingly, we also observed increased PP2A phosphatase activity after treatment with TNF- $\alpha$  (Fig. 3). However, treatment with the MEK inhibitor PD58098 did not induce detectable PARP cleavage, alteration of Akt and p38 phos-

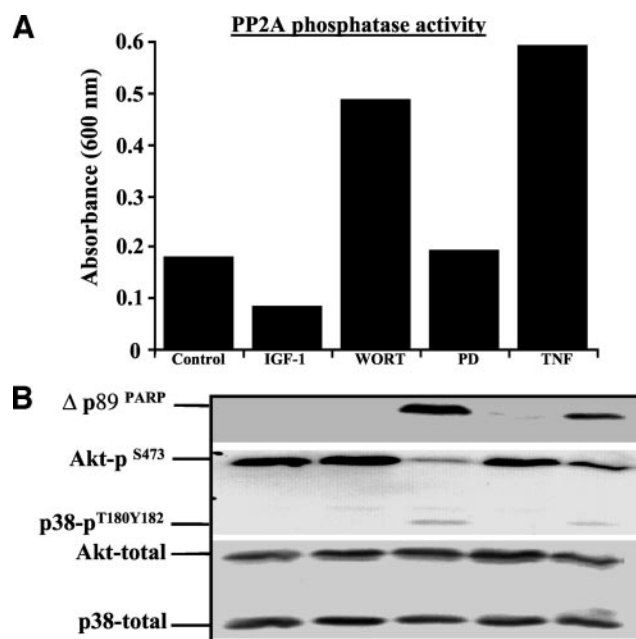


Fig. 3. Enhanced PP2A phosphatase activity in TNF- $\alpha$  induced apoptosis. PP2A phosphatase activity and Western blot analysis of the expression of phospho-Akt, phospho-p38, and PARP cleavage in MDA-MB-231 cells with treatment of insulin-like growth factor-1 (50 ng/mL), TNF- $\alpha$  (50 ng/mL), the phosphatidylinositol 3'-kinase inhibitor Wortmannin (WORT; 0.5  $\mu\text{mol/L}$ ), and the MEK1/2 inhibitor PD98058 (PD; 20  $\mu\text{mol/L}$ ).

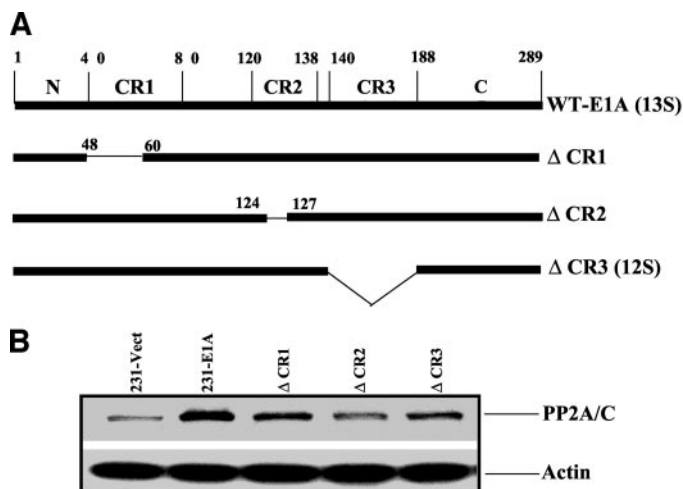


Fig. 4. Conserved domains of E1A required for up-regulation of PP2A/C. *A*, a domain structure and map for deletion mutation of CR1 and CR2. *B*, Western blot analysis of PP2A/C in vector-transfected, or wild-type (WT) E1A and mutant E1A ( $\Delta$ CR1,  $\Delta$ CR2, or  $\Delta$ CR3). Actin was used as a loading control.

phorylation, or alteration of PP2A activity. Although insulin-like growth factor-1 did not dramatically affect Akt or p38 phosphorylation at the 24-hour time point, it slightly reduced PP2A activity (Fig. 3). These results suggest that PP2A may also be involved in TNF- $\alpha$ -induced PARP cleavage and apoptosis. In addition, when Akt activity was blocked by phosphatidylinositol 3'-kinase inhibitor wortmannin, PP2A activity was increased, which correlated with decreased Akt phosphorylation, increased p38 phosphorylation, and PARP cleavage (Fig. 3). Additionally, blocking Akt activation using another phosphatidylinositol 3'-kinase inhibitor, LY249002, also increased PP2A activity (data not shown). Thus, the PP2A activity is also involved in apoptosis induced by the blockade of phosphatidylinositol 3'-kinase-Akt pathway.

**A Conserved Domain of E1A Is Required for Up-Regulation of Protein Phosphatase 2A/C and Sensitization to Drug-Induced Apoptosis.** Because PP2A/C is required for sensitization to drug-induced apoptosis, we then asked whether a deletion mutation of any conserved domain (CR) of E1A, which is required for E1A to sensitize cells to drug-induced apoptosis, also disrupts the ability of E1A to up-regulate PP2A/C expression. In our previous report, we had mapped that among the three conserved domains of E1A, CR2 is associated with E1A-mediated sensitization to drug-induced apoptosis (10). Therefore, we used the same set of wild-type E1A or conserved domain deletion mutant stable cells ( $\Delta$ CR1,  $\Delta$ CR2, and  $\Delta$ CR3) established in MDA-MB-231 cells to test whether the same deletion mutation would affect E1A-mediated up-regulation of PP2A/C (Fig. 4A). As expected, we found that deletion mutation of the CR2 domain impaired the ability of E1A to up-regulate PP2A/C expression, whereas the CR1 or CR3 domain mutant only slightly affected E1A-mediated up-regulation of

PP2A/C in the presence of paclitaxel (Fig. 4B). Thus, this result further supports the requirement of E1A-mediated chemosensitization for up-regulation of PP2A/C.

Taken together, in the current study, we showed that E1A-mediated sensitization to drug-induced apoptosis involves activation of PP2A through up-regulation of PP2A/C expression, which results in activation of p38 and repression of Akt. In addition, activation of PP2A/C is required for E1A-mediated sensitization to drug-induced apoptosis, because blocking PP2A/C expression by a small interfering RNA against PP2A/C reduced drug sensitivity of E1A-expressing cells. Deletion mutation of the conserved domain of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, abolished the ability of E1A to up-regulate PP2A/C (Fig. 4B) and down-regulate Akt activation (10). Thus, by repressing Akt activation through PP2A, E1A up-regulates p38 and facilitates cytochrome *c* release from mitochondria (Fig. 2A), which, in turn, contributes to E1A-mediated sensitization to drug-induced apoptosis.

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