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Retinoic Acid Induced Repression of AP-1 Activity Is Mediated by Protein Phosphatase 2A in Ovarian Carcinoma Cells

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Abstract In previous studies we have shown that *all-trans* retinoic acid (*at*RA)-treatment of the *at*RA-sensitive ovarian carcinoma cell line CA-OV3 repressed AP-1 activity by about 50%, while a similar effect was not observed in the *at*RA-resistant ovarian carcinoma cell line, SK-OV3. These results suggested that the repression of AP-1 activity may be one of the mechanisms by which *at*RA inhibits the growth of *at*RA-sensitive CA-OV3 cells. In the present studies, we investigated further the molecular mechanism by which AP-1 activity is repressed by *at*RA. We show that the repression of AP-1 activity correlates with an increase in JunB protein expression and a decrease in N-terminal phosphorylation of c-Jun. The decrease in N-terminal phosphorylation of c-Jun does not appear to be modulated by JNK or ERK, since their protein expression patterns and kinase activity do not correlate with the repression of AP-1 activity following treatment with *at*RA. However, the activity of the protein phosphatase PP2A was found to increase 24 h following *at*RA treatment in CA-OV3 cells. Moreover, the catalytic subunit of PP2A was found to associate with c-Jun in vivo following *at*RA treatment. Since the inhibition of AP-1 activity following *at*RA treatment of CA-OV3 cells was abolished in the presence of specific PP2A inhibitors, it is likely that PP2A plays an important role in the *at*RA-induced repression of AP-1. J. Cell. Biochem. 96: 170–182, 2005.

Key words: retinoic acid; AP-1; PP2A

Ovarian cancer is a disease that claims the lives of over 14,000 women each year in the United States. This makes it the highest mortality rate of all gynecological cancers. The high incidence of mortality is due to the absence of symptoms until the very late stages of the disease [Daly and Obrams, 1998; Milde-Langosch and Riethdorf, 2003]. Therefore, it is of great importance to devise effective therapies to treat this disease. Retinoid therapy has been used to treat a variety of cancers and has been shown to inhibit the growth of many carcinoma cells, including ovarian tumor cell lines [Harant et al., 1993; Saunders et al., 1995; Chao et al., 1997; Wu et al., 1997, 1998a,b; Sabichi et al., 1998; Pergolizzi et al., 1999]. However, its mechanisms of action are not fully understood.

Retinoids, which are vitamin A derivatives, play a distinctive role in regulation of cell growth and differentiation [Wu et al., 1998b]. It has been shown that retinoids are also able to inhibit epithelial tumor progression in mice and in established cell lines [Wu et al., 1997, 1998a]. We have previously shown that in human ovarian carcinoma cell lines, such as CA-OV3, *all-trans* retinoic acid (*at*RA) inhibits cell growth, DNA synthesis, anchorage-independent colony formation in soft agar, and mediates cell cycle arrest in G_1. In contrast, SK-OV3 cells,

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which are derived from a more aggressive, later stage ascites ovarian tumor, are resistant to this effect. This growth inhibition is mediated by the interaction of *at*RA with its nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR) [Wu et al., 1997].

Many studies have shown that retinoic acid represses the transcriptional activity of activator protein-1 (AP-1) [Schule et al., 1991; Soprano et al., 1996; Zhou et al., 1999; Benkoussa et al., 2002; Lin et al., 2002; Suzukawa and Colburn, 2002]. AP-1 is a transcription factor consisting of a combination of dimers of Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1, Fra2), and CREB/ATF (CREB, ATF2, ATF3, Maf, B-ATF) families of transcription factors [Echlin et al., 2000]. AP-1 is important in regulating the G_0/G_1 transition of the cell cycle. The activation of the c-Jun component of AP-1 depends on posttranslational modification of specific serine and threonine residues, which are phosphorylated by mitogen-activated protein kinases (MAPKs) [Clerk and Sugden, 1997; Chang and Karin, 2001; Cohen and Frame, 2001]. MAPKs are proline-directed serine/threonine kinases, which respond to chemical and physical stresses by connecting cell surface receptor responses to regulatory protein actions [Clerk and Sugden, 1997; Chang and Karin, 2001; Cohen and Frame, 2001]. There are four groups of MAPKs: ERK, JNK, p38 (α, β, γ) and ERK 5 [Binétruy et al., 1991; Clerk and Sugden, 1997; Chang and Karin, 2001]. JNKs (c-Jun N-terminal kinases) phosphorylate Ser-63 and Ser-73 of c-Jun. These phosphorylations allow binding of c-Jun to CBP (CREB binding protein), an adaptor protein which makes the basal transcriptional elements available to c-Jun [Karin, 1995] and enhance its transactivation properties [Binétruy et al., 1991; Fuchs et al., 1997; Chang and Karin, 2001]. On the other hand, ERK 1/2 phosphorylate C-terminal residues Thr-231, Ser-243, and Ser-249, inhibiting c-Jun transcriptional activity by interfering with its association with DNA [Minden et al., 1994; Chang and Karin, 2001]. AP-1 activity can also be regulated by serine/threonine protein phosphatase 2A (PP2A). There is evidence showing that inhibition of PP2A with okadaic acid or I_2^{PP2A}, a potent and specific inhibitor of PP2A, increases DNA-binding and transcriptional activity of AP-1 as well as N-terminal phosphorylation of c-Jun [Black et al., 1991; Al-Murrani et al., 1999].

Our previous studies show that *at*RA-sensitive CA-OV3 cells exhibit approximately a 50% decrease in AP-1 activity as early as 24 h after *at*RA treatment. Conversely, SK-OV3 cells do not show changes in AP-1 activity after similar treatment. Repression of AP-1 activity can be induced in SK-OV3 cells by over-expressing any of the RAR's (α, β, and γ) along with RXR-α (SK-OV3 RARα/RXRα) [Soprano et al., 1996]. Restoration of AP-1 antagonism in these cells is accompanied by conversion of the growth responses from *at*RA resistance to *at*RA sensitivity. Additionally, we have shown that PP2A protein levels and activity increase following *at*RA treatment of CA-OV3 cells [Vuocolo et al., 2003]. In the present studies, we investigated potential mechanisms by which *at*RA might repress AP-1 activity. Our results show that *at*RA treatment of CA-OV3 cells leads to an increase in JunB expression and a decrease in c-Jun N-terminal phosphorylation, concomitant with the repression of AP-1 activity. Moreover, it appears that induction of PP2A by *at*RA plays an important role in this reduction in c-Jun phosphorylation.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Growth Assays

CA-OV3 and SK-OV3 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured as previously described [Tairis et al., 1995; Wu et al., 1998b]. All stock cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100-U/ml penicillin and streptomycin. They were maintained in an incubator at high humidity, 5% CO_2 and 37°C. The cells were passaged twice every week. Cell growth and viability were determined by plating cells at a density of 1×10^5 cells in 100 mm dishes. Retinoic acid (*at*RA) treatments were done on days 1, 3, and 5 after plating the cells. Cells were removed from tissue culture plates with trypsin on day 6 following treatment. Cell viability was determined by trypan blue exclusion. Cells were counted using a hemacytometer.

Reagents

Anisomycin, PD98059, okadaic acid, endothall, and fostriecin were obtained from Calbiochem (La Jolla, CA). Anisomycin is an activator of MAPK/SAPK signaling pathways [Barancik

et al., 1999]. PD98059 (10 μM stock) was used in the assays requiring ERK inhibition [Westermarck et al., 2001; Bost et al., 2002]. Okadaic acid (10 μM stock), endothall (10 μM stock), and fostriecin (1 μM stock) were used in the assays requiring inhibition of serine/threonine phosphatases. Okadaic acid inhibits PP2A at a concentration of 10 nM, and PP1 and PP2A at a concentration of 1 μM in vitro [Sasaki et al., 1994; Walsh et al., 1997; Laidley et al., 1999]. Endothall and fostriecin are specific PP2A inhibitors [Li et al., 1993; Thiery et al., 1999].

*at*RA was supplied by Hoffman La Roche, Inc. (Nutley, NJ). Stock solutions of 1 mM were prepared in ethanol and stored at −20°C. All procedures involving the use of *at*RA were carried out under subdued light. Fresh stocks were prepared every 2 weeks.

Total Protein Isolation and Western Blot Analysis

To determine protein expression following the specified treatments, the cells were plated in 100 mm dishes at a density of 5×10^6 cells/plate. Treatments were carried out for a maximum time of 24 h. The cells were treated as indicated in each figure legend. To isolate total protein, the cells were washed twice with ice cold PBS and harvested by scraping. The cells were pelleted and resuspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, pH 8.0, 1% NP-40) supplemented with protease inhibitors: aprotinin, leupeptin, trypsin inhibitor (10 μg/ml each, Sigma, Atlanta, GA), PMSF (1 mM, Sigma), and pepstatin A (1 μg/ml, Sigma). Usually 1 ml of lysis buffer was added per 10^7 cells and incubated on ice for 30 min. The cell lysate was vortexed two or three times during the incubation and centrifuged at 14,000 rpm for 8 min at 4°C. The supernatant was saved. Protein concentration was determined using the Bradford assay (Bio Rad, Hercules, CA). Approximately 100 μg of protein was analyzed on 10% SDS–PAGE gels at 120 V/35 mA for 3–5 h. The separated proteins were transferred to a PVDF membrane (Millipore, Bedford, MA) by electroblotting, using a Hoeffer apparatus.

The membranes were incubated on a rocking platform in blocking buffer (5% non-fat dry milk in TBST: 20 mM Tris, pH 7.6, 150 mM NaCl, and 0.01% Tween-20) at room temperature for 1 h or at 4°C overnight. The membranes were then incubated in primary antibody diluted 1:500 (p-c-Jun, p-JNK, and p-ERK) or 1:1,000 (c-Jun, c-Fos, ERK1, JNK2, actin) in blocking buffer for 1 h at room temperature, followed by three washes with TBS plus 0.1% Tween-20 for 10 min at room temperature. After washing, the membranes were incubated with the corresponding HRP-conjugated IgG secondary antibodies, at a dilution of 1:2,000, in blocking buffer for 45 min at room temperature. The membranes were washed three times with TBST. Protein detection was performed using ECL™ (Amersham Life Sciences, Buckinghamshire, England).

The primary antibodies used were: c-Jun(H-79), c-Fos(H-125), JunD(329), JunB(N-17), p-c-Jun(KM-1), JNK2(FL), p-JNK(G-7), ERK1(C-16), PP2A(c-20), actin(I-19), obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-MAP Kinase/ERK1/2(Y180), anti-PP2A, B subunit, clone 2G9 and anti-phospho MBP were obtained from Upstate Biotechnology (Lake Placid, NY). The secondary antibodies used were: HRP-conjugated anti-mouse and anti-rabbit (Amersham Life Sciences), rabbit anti-sheep (Upstate Biotechnology), and donkey anti-goat (Santa Cruz Biotechnology, Inc.).

Immunoprecipitation

Total protein isolates were prepared as described above. Approximately 500 μg of protein was incubated on a rocking platform with 4 μg of the corresponding antibody for at least 3 h at 4°C. The antibody/antigen complexes were collected by incubating with 40 μl of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.), for at least 1 h at 4°C. The immunoprecipitates were eluted from the beads using 60 μl of sample buffer (1.2 mM Tris, 20% glycerol, 2% SDS, 10 μg/ml bromophenol blue, adjust to pH 6.8) followed by boiling for 5 min. The samples were microfuged to collect the supernatants and analyzed by Western blot.

JNK and ERK Kinase Activity Assays

To determine the activity of JNK and ERK kinases following *at*RA treatments, the cells were washed twice with ice cold PBS. Nuclear protein was isolated as previously described [Wu et al., 1997]. Briefly, the cell pellet was resuspended in five times its volume with lysis buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 0.2 mM EDTA, plus protease inhibitors, as previously cited). The suspension was incubated on ice for 10 min and lysed with a Dounce

homogenizer. The suspension was then centrifuged and the nuclei resuspended in 1 ml of lysis buffer B (20 mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol, 0.2 mM EDTA, plus protease inhibitors). The tube was incubated at 4°C for 30 min on a rotating platform and then centrifuged at full speed for 15 min at 4°C. The supernatant was collected for protein concentration determination using the Bradford assay (Bio Rad). Approximately 100 μg of protein was immunoprecipitated with 4 μg of JNK2 (FL) or ERK1 (C-16) antibodies, as described. The collected complexes were used with the non-radioactive SAPK1a/JNK or MAP Kinase assay kits (Upstate Biotechnology), as indicated by the manufacturer's instructions. Briefly, the immunoprecipitates were incubated in assay dilution buffer with their corresponding substrates c-Jun-GST (for SAPK/JNK assay) and MBP (for MAP Kinase assay) and Mg^{2+}/ATP cocktail for 30 min at 30°C in a shaking incubator. The kinase reaction was stopped by adding sample buffer, followed by boiling for 5 min. The samples were microfuged to collect the supernatants, which were separated on a 10% SDS–PAGE gel for Western blot analysis to detect the phosphorylation state of the substrates, using p-c-Jun (KM-1) and anti-phospho MBP antibodies, respectively.

Serine/Threonine Phosphatase Activity Assay

CA-OV3 cells were plated at a density of 5×10^6 cells in 100 mm dishes and treated with phosphatase inhibitors 30 min prior to *at*RA treatments. Cells were harvested 24 h following *at*RA treatments. Phosphatase activity was measured from total protein isolates using the non-radioactive Serine/Threonine Phosphatase Assay System (Promega, Madison, WI) as indicated by the manufacturer's instructions. The reactions were performed in a 96-well, flat-bottomed plate, using 2.5 μg of protein in a total volume of 50 μl per sample. The suggested reaction buffer (250 mM imidazole, pH 7.2, 1 mM EGTA, 0.1% β-mercaptoethanol, 0.5 mg BSA), and the phosphopeptide substrate used in this reaction were optimized to measure PP2A activity and result in poor measurements for the activity of PP1, another major phosphatase. The assay plate was incubated in a water bath at 30°C for 30 min, followed by addition of 50 μl of molybdate dye/additive cocktail and incubation at room temperature for 15 min to stop the reaction. The assay determines the amount of released phosphates by measuring the absorbance of a complex formed by molybdate: malachite green: phosphate at a wavelength of 620 nm [Ekman and Jager, 1993]. The absorbance was measured using a plate reader (Bio Rad).

AP-1 Activity Assays

AP-1 activity assays were performed as previously described [Soprano et al., 1996]. CA-OV3 cells were plated at a density of 5×10^5 cells in 60 mm dishes. The cells were transfected the next day with 1 μg pCMV-βgal, 4 μg pCAT-AP1, and 5 μg empty pSG5 vector using the Ca^{2+} phosphate method. The pCAT-AP1 reporter construct contains three repeats of the AP-1 consensus sequence (5′-GACTCAT-3′) inserted upstream of the promoter driving the chloramphenicol acetyl transferase gene (*CAT*). Twenty-four hours later, the cells were treated with PD98059, endothall or fostriecin, 30 min prior to *at*RA treatment. Following an additional 24 h, the cells were harvested and assayed for β-galactosidase [Eustice et al., 1991] and CAT [Seed and Sheen, 1988] activities. The efficiency of transfection was determined by β-galactosidase, which was then used to normalize the CAT activity. The percent AP-1 activity was calculated setting the ethanol treated control as 100% activity.

RESULTS

Effects of *at*RA Treatment on Protein Expression of Select AP-1 Dimerization Partners in Ovarian Carcinoma Cell Lines

We have previously reported that CA-OV3 cells exhibit 50%–60% growth inhibition following *at*RA treatment, while SK-OV3 cells are resistant to this growth inhibition [Wu et al., 1998b]. Also our previous studies showed that *at*RA treatment induced repression of AP-1 activity to about 50% in *at*RA sensitive CA-OV3 cells, by 24 h after treatment, but did not repress AP-1 activity in *at*RA-resistant SK-OV3 cells [Soprano et al., 1996]. AP-1 activity can be regulated by the amount of AP-1 protein components in the cell [Chiu et al., 1989; Angel and Karin, 1991]. As a first approach to understanding the mechanism responsible for the repression of AP-1 activity by *at*RA, we initially determined the levels of protein expression of members of the Jun family (c-Jun, JunB, and JunD) and c-Fos, in *at*RA-sensitive CA-OV3, at

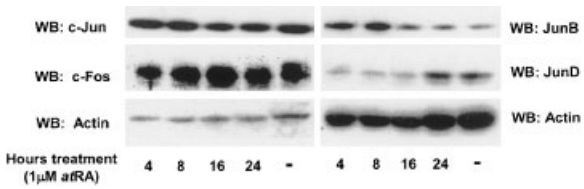

Fig. 1. Protein levels of select AP-1 dimerization partners following *all-trans* retinoic acid (*at*RA) treatment. Cells were treated with 1 μM *at*RA or ethanol control as indicated. Total protein isolates from CA-OV3 ovarian carcinoma cells were used for Western blot analysis using antibodies recognizing c-Jun and c-Fos (**left panels**) or JunB and JunD (**right panels**). Reprobing of the membranes with an antibody recognizing actin was used as a loading normalizer.

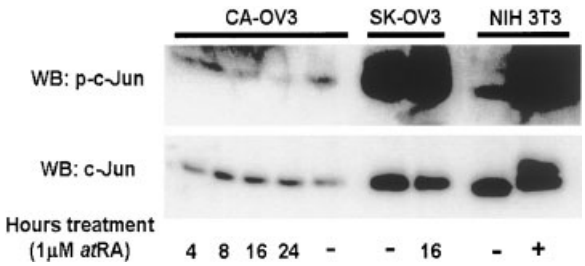

Fig. 2. Phosphorylation state of c-Jun following *at*RA treatment. Western blot of total protein isolates from CA-OV3 and SK-OV3 ovarian carcinoma cell lines, using antibodies recognizing c-Jun phosphorylated on Ser-63 (p-c-Jun), and total c-Jun (c-Jun), to measure total loading. Cell lines were treated with vehicle (−) or 1 μM *at*RA for the indicated times. NIH 3T3 cells were treated with anisomycin 30 min prior to harvesting, and the total protein isolates were used as positive control for the induction of MAPKs and their substrates (+).

various times following treatment with *at*RA. We found that c-Jun protein levels did not change in *at*RA-sensitive CA-OV3 cells after *at*RA treatment (Fig. 1). c-Fos protein levels fluctuated in CA-OV3 cells following *at*RA treatment, however they did not exceed protein levels of the control sample more than twofold as analyzed by densitometry (data not shown).

In *at*RA-sensitive CA-OV3 cells, JunB protein levels were increased at 4 and 8 h after *at*RA treatment, and returned to control levels by 16 h following *at*RA treatment, when normalized to actin protein levels. JunD protein levels did not change significantly with treatment, as revealed by densitometric analysis (data not shown).

*at*RA Induces a Decrease in Ser-63 Phosphorylation of c-Jun in *at*RA-Sensitive CA-OV3 Cells

Another mechanism by which AP-1 activity is regulated is N-terminal phosphorylation of c-Jun on serine residues 63 and 73 (Ser-63 and Ser-73), a modification that allows binding to CBP and accessibility to the transcriptional machinery [Karin, 1995]. The top panel on Figure 2 shows that treatment of *at*RA-sensitive CA-OV3 cells with *at*RA leads to a decrease in c-Jun phosphorylation on Ser-63 between 16 and 24 h after treatment. These results were not observed in the *at*RA-resistant cell line SK-OV3 (Fig. 2). This experiment suggests that the repression of AP-1 activity observed in CA-OV3 cells may be due at least in part to a reduction in N-terminal phosphorylation of c-Jun.

Treatment With *at*RA Does not Change the Protein Levels and Activity of JNK

Since *at*RA treatment was shown to decrease the N-terminal phosphorylation of c-Jun on Ser-63, we decided to examine whether this change was due to a decrease in the protein levels and/or activity of Jun-N-terminal kinase (JNK). JNK has been shown to phosphorylate Ser-63 and Ser-73 of c-Jun, increasing its transcriptional activity [Minden et al., 1994b]. As shown in Figure 3A, treatment with *at*RA slightly increased JNK protein levels at 8, 16, and 24 h after treatment in *at*RA-sensitive CA-OV3 cells, although the increase was less than twofold, compared to the control sample, as determined by densitometry. The phosphorylation of JNK on Thr-183 and Tyr-185 activates its kinase activity [Kyriakis et al., 1994]. Western blot analysis using an antibody specific to phosphorylated Thr-183 and Tyr-185 of JNK, showed no changes after treatment with *at*RA (Fig. 3A). Additionally, we could not detect any changes in JNK activity using a SAPK/JNK activity assay, further confirming that JNK activity is not altered by treatment with *at*RA in sensitive cells (Fig. 3B).

*at*RA Induces ERK Activity in CA-OV3 Cells

The ability of c-Jun to bind DNA and thus contribute to the induction of AP-1 responsive genes is also regulated by the phosphorylation of Thr-231, Ser-243, and Ser-249, located on the DNA binding domain. It has been demonstrated that ERK1 and ERK2 are the kinases that phosphorylate these residues and consequently inhibit AP-1 activity [Papavassiliou et al., 1995]. Therefore, we analyzed the effects of *at*RA treatment on ERK protein levels and activity in ovarian carcinoma cells. Treatment with *at*RA did not alter the protein expression of ERK1 and ERK2 in *at*RA-sensitive CA-OV3

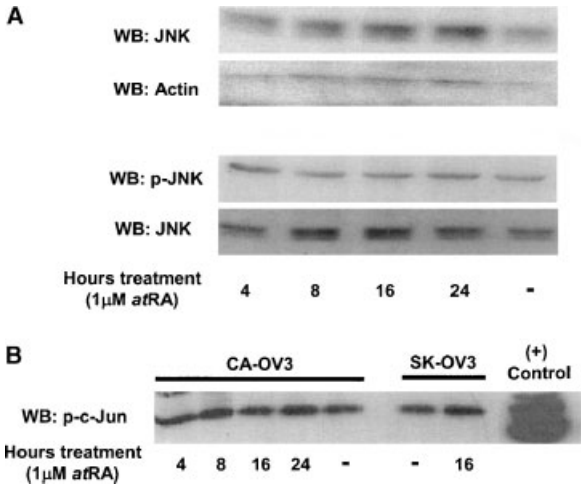

Fig. 3. Analysis of protein expression and kinase activity of JNK, following *at*RA treatment. Total protein isolates from CA-OV3 cells were analyzed by Western blot. Cells were treated with 1 μM *at*RA for the indicated times. **A**: Antibodies recognizing JNK and JNK phosphorylated on Thr-183 and Tyr-185 were used for detection. Phosphorylation of these two residues is required for kinase activity. **B**: Kinase activity assay for JNK. Cells were treated with 1 μM *at*RA for the indicated times and the nuclear proteins were isolated and assayed for SAPK/JNK activity using c-Jun-GST as a substrate. The reaction was carried out as indicated in the ''Materials and Methods.'' The product was analyzed by Western blot using an antibody specific to c-Jun phosphorylated on Ser-63. CA-OV3 and SK-OV3 cells were treated as indicated. Recombinant active JNK incubated with the kinase reaction cocktail was used as a positive control.

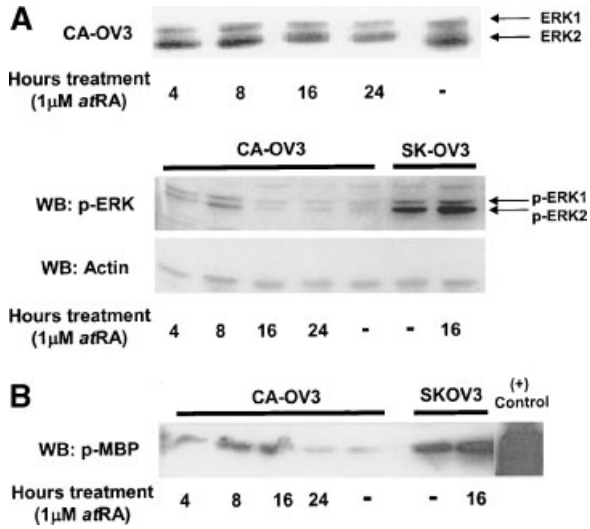

Fig. 4. Analysis of protein expression and kinase activity of ERK, following *at*RA treatment. CA-OV3 and SK-OV3 ovarian carcinoma cells were treated with 1 μM *at*RA for the indicated times. **A**: Total protein from the indicated cells lines was isolated and analyzed by Western blot using an antibody recognizing ERK1/ERK2 (**upper panel**) or ERK phosphorylated on Tyr-180 (**lower panel**). Phosphorylation of this residue is required for kinase activity. **B**: Kinase activity assay for ERK. Cells were treated with 1 μM *at*RA for the indicated times and the nuclear proteins were isolated and assayed for MAPK activity using MBP as a substrate. The reaction was carried out as indicated in the ''Materials and Methods.'' The product was analyzed by Western blot using an antibody specific to phosphorylated MBP. CA-OV3 and SK-OV3 cells were treated as indicated. GST-purified thiophosphorylated p42 MAPK incubated with the kinase reaction cocktail was used as a positive control.

cells (Fig. 4A, top panel). Similar to JNK, the kinase activity of ERK depends on the phosphorylation of a key tyrosine residue (Tyr-180) [Wilsbacher et al., 1999]. Western blot analysis of ERK using an antibody recognizing ERK phosphorylated on Tyr-180 (p-ERK) showed an increase in phosphorylated ERK between 4 and 8 h, followed by a reduction to basal levels at 16 h following *at*RA treatment (Fig. 4A, bottom panel). In *at*RA-resistant SK-OV3 cells, the levels of phosphorylated ERK were slightly altered by *at*RA treatment at 16 h following *at*RA treatment. Additionally, it appears that the basal levels of p-ERK are higher in *at*RA-resistant SK-OV3 cells compared to *at*RA-sensitive CA-OV3 cells. Analysis of ERK activity in *at*RA-sensitive CA-OV3 cells, showed an increase in ERK kinase activity at 4, 8, and 16 h. ERK activity returned to basal levels by 24 h following treatment. No changes in ERK activity were detected in *at*RA-resistant SK-OV3 cells following *at*RA treatment (Fig. 4B). Taken together, these results suggest that the repression of AP-1 activity by *at*RA could be mediated by an increase in ERK activity and subsequent phosphorylation of the DNA binding domain of c-Jun. This post-translational modification could hinder the ability of c-Jun to bind to AP-1 consensus sequences, resulting in a reduced AP-1 activity in the cells.

Inhibition of ERK Activity Does not Reverse *at*RA-Induced Repression of AP-1 Activity in CA-OV3 Cells

To correlate the repression of AP-1 activity by *at*RA in CA-OV3 cells to the phosphorylation of serine and threonine residues on the DNA binding domain of c-Jun by ERK, we assayed AP-1 activity following treatment with PD98059 and *at*RA. PD98059 has been shown to inhibit ERK when used at a concentration of 20 μM in human embryonal fibroblasts [Westermarck et al., 2001]. Treatment with 1 μM *at*RA repressed AP-1 activity by about 35%, compared to

control cells. Treatment with 15 μM PD98059 alone or following *at*RA treatment did not change AP-1 activity, compared with control or *at*RA treated cells. Treatment of cells with a higher dose of PD98059 (30 μM), actually increased AP-1 activity twofold, and did not alter the effect of *atR*A on repression of AP-1 activity (Fig. 5). These results suggest that the mechanism through which ERK modulates AP-1 activity may be different from that through which *at*RA mediates repression of AP-1 activity in *at*RA-sensitive CA-OV3 cells.

Reduction of c-Jun N-Terminal Phosphorylation Correlates With the Induction of PP2A Activity Following *at*RA Treatment of CA-OV3 Cells

AP-1 activity can also be regulated by the serine/threonine phosphatase 2A (PP2A), which dephosphorylates c-Jun on the N-terminal residue Ser-63 [Black et al., 1991; Al-Murrani et al., 1999]. We have previously shown that *at*RA treatment of CA-OV3 cells induces an increase in protein levels and activity of the catalytic subunit of PP2A (PP2Ac) [Vuocolo et al., 2003]. In the present experiments we observed a 2.5-fold increase in phosphatase activity in CA-OV3 cells 24 h following *at*RA treatment (Fig. 6A). The use of endothall, a specific inhibitor of PP2A, produced a concentration-dependent reduction in phosphatase activity in both control and *at*RA-treated samples (Fig. 6B), suggesting that at least a portion of the increase in phosphatase activity by *at*RA corresponds to an increase in PP2A. To begin to determine if the increase in PP2A activity modulates the reduction of phosphorylation of c-Jun on Ser-63 following *at*RA treatment, we treated CA-OV3 cells with phosphatase inhibitors and determined the effect on phosphorylation of c-Jun at Ser-63. As shown in Figure 6C, the inhibition of phosphatase activity (PP1 and PP2A) with 1 μM okadaic acid in the presence of *at*RA partially restored the protein levels of c-Jun phosphorylated on Ser-63 to those of control cells. The inhibition of PP2A activity by treatment with 10 μM of the PP2A-specific inhibitor, endothall, in the presence of *at*RA, restored the protein levels of c-Jun phosphorylated on Ser-63 to those of control levels (Fig. 6D). Moreover, treatment with 25 μM of endothall increased the protein levels of c-Jun phosphorylated on Ser-63 above basal levels in both control and *at*RA-treated cells. These data strongly suggest that PP2A is able to modulate the dephosphorylation of residue Ser-63 of c-Jun following *at*RA treatment.

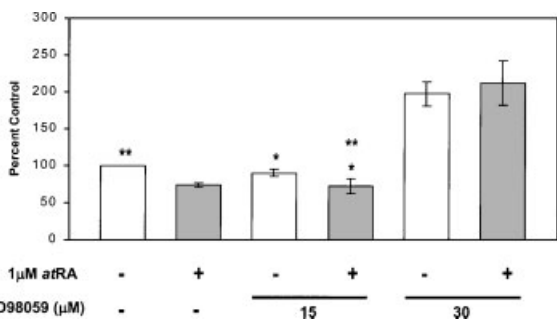

Fig. 5. Effects of ERK inhibition on AP-1 transcriptional activity, following *at*RA treatment in ovarian carcinoma cells. CA-OV3 cells were transiently transfected with 4 μg pCAT-AP1, 1 μg pCMV-βgal, and 5 μg pSG5. Twenty-four hours after transfection, cells were treated with the indicated concentrations of PD98059, a specific inhibitor of ERK activity, followed by 1 μM *at*RA treatment 30 min later. Cells were harvested 24 h after addition of *at*RA and assayed for CAT activity. β-galactosidase activity was used to measure transfection efficiency. The experiments were performed in triplicate, and the values presented represent the mean values for each sample with their corresponding standard errors. Statistical analysis using the paired *t*-test ($P < 0.05$) was made using Instat™ software from GraphPad. (*) Indicates there is a statistically significant difference between the mean values of the samples treated with 15 μM PD98059 alone versus the samples treated with 15 μM PD98059 plus 1 μM *at*RA. (**) Indicates there is a statistically significant difference between the mean values of the samples treated with 15 μM PD98059 plus 1 μM *at*RA versus control.

c-Jun Interacts With the Catalytic Subunit of PP2A in CA-OV3 Cells

To support the proposed mechanism of the dephosphorylation of residue Ser-63 of c-Jun by PP2Ac in CA-OV3 cells, we immunoprecipitated c-Jun from protein extracts obtained from CA-OV3 cells that had been treated with *at*RA for 16 and 24 h. A Western blot using an antibody specific for PP2Ac showed that PP2Ac associates with c-Jun in vivo (Fig. 7). The association between c-Jun and the catalytic subunit of PP2A appears to be greater than the association of c-Jun with the B-regulatory subunit, PR56. However, this may reflect the fact that the PR56 B-regulatory subunit is most highly expressed in brain and is not highly expressed in ovary [Janssens and Goris, 2001]. Moreover, the modest signal with PR56 may be a consequence of the nature of the PR56 antibody used for the assay. Finally, it should be noted, however, that the interaction between c-Jun and PP2Ac did

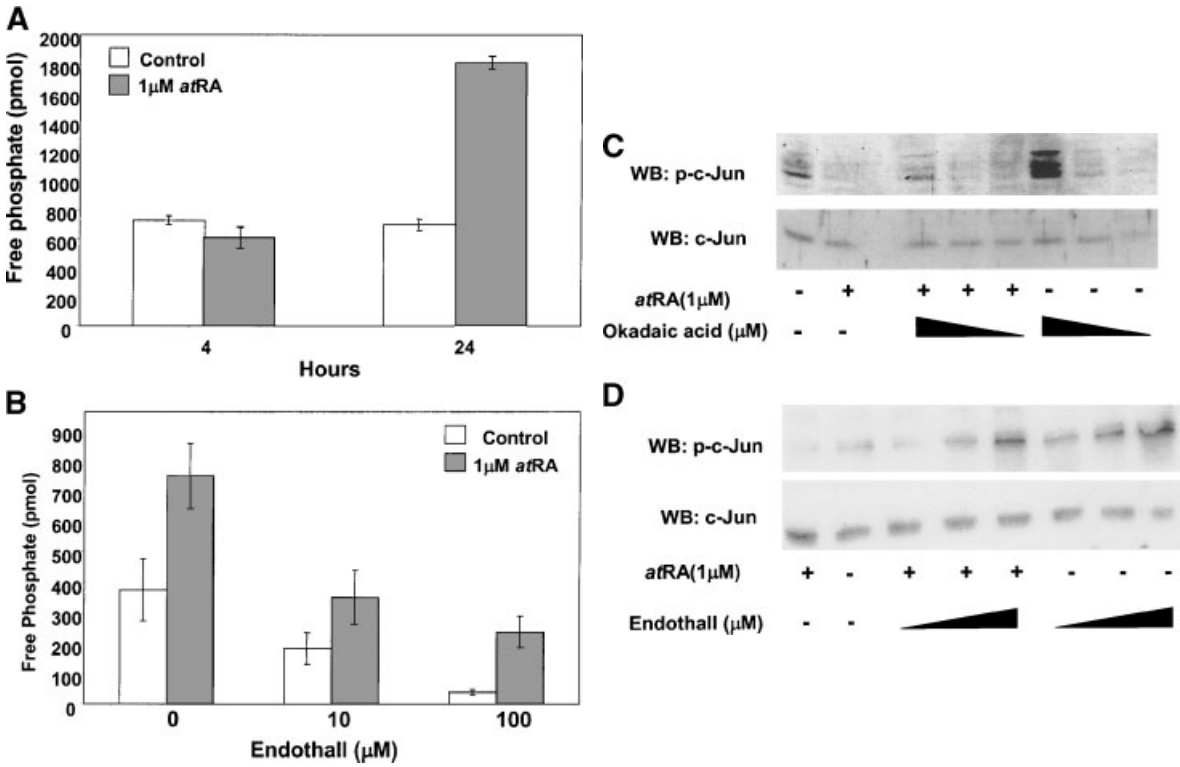

Fig. 6. Reduction of c-Jun N-terminal phosphorylation correlates with the induction of PP2A activity following *at*RA treatment. **A**: Phosphatase activity assay using total protein extracts from CA-OV3 cells treated with 1 μM *at*RA for the indicated time points. Phosphatase activity was measured as free phosphate released from a phosphopeptide substrate specific for Ser/Thr phosphatases and a reaction buffer optimized for PP2A activity, as described in the ''Materials and Methods.'' **B**: Phosphatase activity assay was performed on CA-OV3 cells treated with 1 μM *at*RA for 24 h. Total protein was isolated and phosphatase activity was measured as previously described. To determine the specificity of the inhibition of phosphatase induced by PP2A, endothall, a specific PP2A inhibitor ($IC_{50} = 90$ nM), was added directly to the reaction cocktail. The experiments in (A) and (B) were performed in triplicate, and the values presented represent the mean values for each sample with their corresponding standard errors. **C**, **D**: CA-OV3 cells were treated with 1 μM *at*RA and/or decreasing concentrations (1, 0.1, 0.01 μM) of okadaic acid (C) or increasing concentrations (5, 10, 25 μM) of endothall (D), for 24 h as indicated. Total protein was isolated and analyzed by Western blot with an antibody recognizing c-Jun phosphorylated on Ser-63.

not appear to be enhanced or reduced by treatment with *at*RA.

Inhibition of PP2A Reverses the Repression of AP-1 Activity Induced by *at*RA in CA-OV3 Cells

To demonstrate that the repression of AP-1 activity induced by *at*RA is a function of the increase in PP2A activity and subsequent dephosphorylation of c-Jun on Ser-63, we measured AP-1 activity in CA-OV3 cells treated with *at*RA in combination with the PP2A specific inhibitors, endothall or fostriecin. As shown in Figure 8A, treatment with endothall alone did not affect the basal AP-1 activity. When the cells were treated with 25 μM endothall and *at*RA for 24 h, AP-1 activity was restored to control levels. Similar results were obtained in an assay using fostriecin, another specific inhibitor of PP2A (Fig. 8B). Treatment with either 5 or 25 μM of fostriecin, a potent inhibitor of PP2A, did not affect basal AP-1 activity. However, these concentrations, together with 1 μM *at*RA were able to restore AP-1 activity to those of control levels. Together these results strongly suggest that the increase in PP2A activity following *at*RA treatment reduces the N-terminal phosphorylation of c-Jun, resulting in the repression of AP-1 transcriptional activity.

DISCUSSION

A correlation between growth inhibition and repression of AP-1 activity in ovarian carcinoma cell lines has been previously demonstrated. AP-1 activity was repressed by ~50% following *at*RA treatment in ovarian carcinoma cells that are growth inhibited by *at*RA [Soprano et al.,

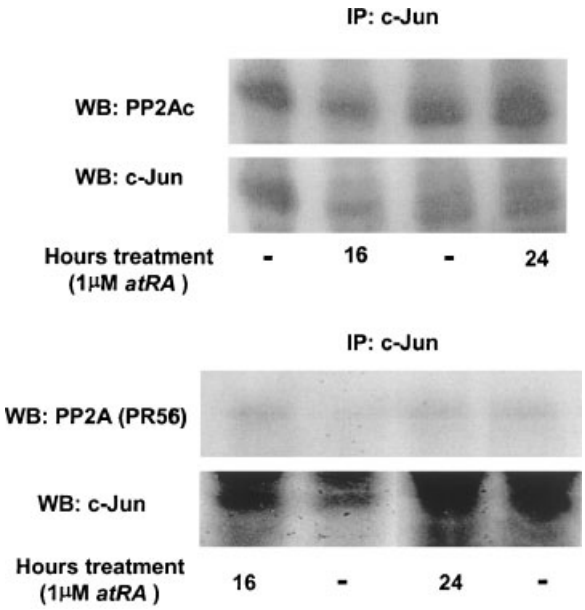

Fig. 7. PP2Ac and c-Jun associate in vivo. Immunoprecipitation of total protein extracts from CA-OV3 cells using an antibody specific to c-Jun, followed by Western blot using antibodies recognizing the catalytic subunit of PP2A (PP2Ac) and B regulatory subunit (PR56). The nitrocellulose membrane was stripped and reprobed with an antibody to c-Jun, to verify total protein loading.

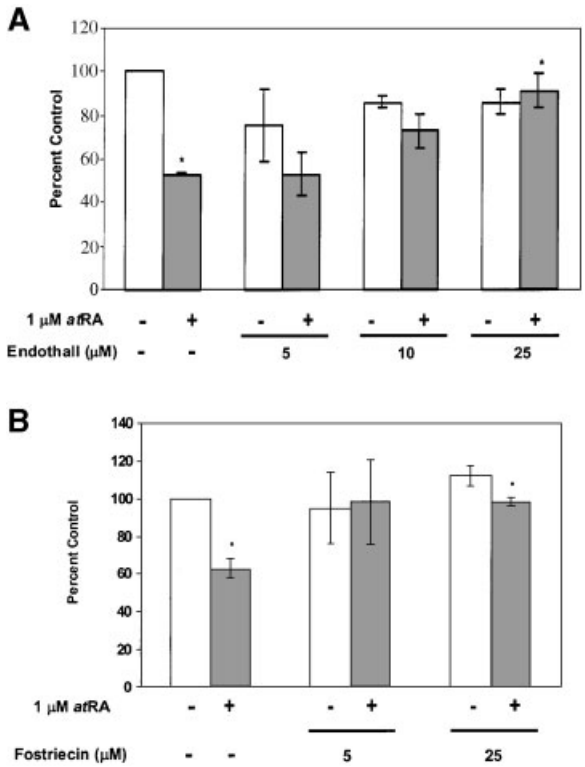

Fig. 8. Effects of PP2A inhibition on AP-1 transcriptional activity, following *at*RA treatment in ovarian carcinoma cells. CA-OV3 cells were transiently transfected with 4 μg pCAT-AP-1, 1 μg pCMV-βgal, and 5 μg pSG5. Twenty-four hours after transfection, cells were treated with the indicated concentrations of endothall (**A**) or fostriecin (**B**), followed by 1 μM *at*RA 30 min later. Cells were harvested 24 h after addition of *at*RA and assayed for CAT activity. β-galactosidase activity was used to measure transfection efficiency. The experiments were performed in triplicate, and the values presented represent the mean values for each sample with their corresponding standard errors. Statistical analysis using the paired *t*-test ($P < 0.05$) was made using Instat™ software from GraphPad. (*) Indicates there is a statistically significant difference between the mean values of the samples treated with 1 μM *at*RA alone, versus the samples treated with phosphatase inhibitor plus 1 μM *at*RA.

1996]. The purpose of this report was to elucidate the mechanisms through which *at*RA represses AP-1 activity in ovarian carcinoma cells. Several mechanisms for *at*RA induced repression of AP-1 activity have been suggested, and vary depending on the cell systems studied. For example, there is evidence that liganded RAR sequesters AP-1 components and inhibits the dimerization of c-Jun to c-Fos [Schule et al., 1991; Pfahl, 1993; Zhou et al., 1999; Suzukawa and Colburn, 2002]. Other evidence shows that *at*RA represses AP-1 activity by inhibiting the Jun N-terminal kinase (JNK) signaling pathway [Caelles et al., 1997; Lee et al., 1999; Chung et al., 2002]. Another suggested mechanism involves the induction of transcription and protein expression of JunB and Fra, which have been described as AP-1 components acting as negative regulators of AP-1 activity [Benkoussa et al., 2002]. Furthermore, it has been suggested that AP-1 activity is repressed by competitive association of liganded RAR to the co-activator CREB binding protein (CBP). However, this evidence has been disproved by indication that the RAR domain capable of inhibiting AP-1 activity does not interact with CBP [Kamei et al., 1996; Lee et al., 1998; Lin et al., 2002].

We have tested a number of potential mechanisms which could be responsible for repression of AP-1 activity by *at*RA treatment. Our results suggest that two mechanisms might contribute to *at*RA-induced anti-AP-1 activity: (1) induction of the protein phosphatase PP2A, leading to the dephosphorylation of the amino terminal of c-Jun; and (2) increased expression of JunB which may act as an inhibitor of AP-1 by sequestering c-Jun in dimeric complexes which bind weakly to AP-1 sites resulting in poor transcriptional transactivation.

AP-1 activity can be regulated by the amount of its components available in the cell. We found

that c-Jun and c-Fos protein levels did not change significantly in the *at*RA-sensitive cell line CA-OV3 following treatment. These results coincide with those reported by Kaiser et al. [1999] in the *at*RA-sensitive pancreatic carcinoma cell line DAN-G. We did observe, however, that there was an inverse proportion of c-Jun and c-Fos protein expression between the *at*RA-sensitive CA-OV3 cells and the *at*RA-resistant SKOV3 cells. In SK-OV3 cells, c-Fos protein levels appeared lower than those detected in CA-OV3 cells, whereas c-Jun protein levels appeared much higher in SK-OV3 cells. It is possible that one of the reasons why SK-OV3 cells do not exhibit repression of AP-1 activity, and are resistant to *at*RA induced growth inhibition, is due to an excess of c-Jun protein. AP-1 can be comprised of either c-Jun homodimers or c-Jun/c-Fos heterodimers [Angel et al., 1987; Mechta-Grigoriou et al., 2001]. Therefore, it is plausible that the excess of c-Jun protein overcomes the pathways of AP-1 repression by *at*RA. Although this does not fully explain the lack of repression of AP-1 activity in *at*RA-resistant SK-OV3 cells, since c-Jun/c-Fos heterodimers form more stable dimers than c-Jun homodimers [Shaulian and Karin, 2001].

c-Jun can form AP-1 dimers with JunB and JunD. In *at*RA-sensitive CA-OV3 cells JunB protein levels were increased between 4 and 16 h following *at*RA treatment. JunB has been described as a negative regulator of AP-1 activity. JunB is less efficient at DNA-binding and transactivation of the AP-1 responsive genes. It is associated with cell cycle inhibition and quiescence, represses the cyclin D-1 promoter and suppresses transformation [Bakiri et al., 2000; Passegue and Wagner, 2000; Mechta-Grigoriou et al., 2001]. Furthermore, there is evidence that RARs induce JunB [Benkoussa et al., 2002]. Thus, it is possible that the changes in JunB levels elicited by *at*RA treatment in CA-OV3 cells play a role in the repression of AP-1 activity.

AP-1 activity can also be regulated by post-translational modifications, such as phosphorylation of key serine and threonine residues. These modifications are elicited by the activities of Jun N-terminal kinase (JNK) and the extracellular signal-regulated kinase (ERK), which phosphorylate the N-terminal transactivation domain and the DNA-binding domain of c-Jun, respectively. The phosphorylation of c-Jun on Ser-63 and Ser-73, located on the N-terminal transactivation domain, enables c-Jun to bind CBP and access the transcriptional complexes [Karin, 1995; Benkoussa et al., 2002]. Our results show a decrease in protein levels of c-Jun phosphorylated on Ser-63 in *at*RA-sensitive CA-OV3 cells following treatment. This decrease was detected between 16 and 24 h after treatment, an event which is concomitant with the time point in which the repression of AP-1 activity was detected in these cells following *at*RA treatment [Soprano et al., 1996]. The decrease in protein levels of c-Jun phosphorylated on Ser-63 was not observed in *at*RA-resistant cell lines SK-OV3. It should be noted that most of the c-Jun protein detected by Western blot analysis in SK-OV3 cells, was phosphorylated on Ser-63 (data not shown), and that this phosphorylation state could not be reduced by *at*RA treatment. This occurrence could also explain the inability of *at*RA to repress AP-1 activity and consequently the growth of SK-OV3 cells.

Since *at*RA has been shown to repress AP-1 activity through the inhibition of JNK signaling pathways, and JNK binds to c-Jun, phosphorylating Ser-63 and Ser-73 in response to various stimuli [Minden et al., 1994; Lee et al., 1999], we decided to test the effects of *at*RA treatment on JNK protein levels and activity. Surprisingly, we could not detect any changes in JNK protein levels or its activity following *at*RA treatment in either *at*RA-sensitive or *at*RA-resistant cell lines. This contrasts with findings by two other groups in different cell model systems stating that JNK activity is inhibited by *at*RA treatment in sensitive cells [Caelles et al., 1997; Lee et al., 1999]. Minden et al. [1994b] demonstrated that ERK phosphorylates key serine and threonine residues (Thr-231, Ser-243, and Ser-249) located on the C-terminal DNA-binding domain, an event which inhibits DNA-binding and consequently the activity of AP-1. Therefore, we assayed the effects of *at*RA treatment on ERK protein levels and activity. Our results illustrate that ERK activity, but not its protein levels, is increased between 4 and 16 h following *at*RA treatment in *at*RA-sensitive CA-OV3 cells. This would agree with our hypothesis that *at*RA induces the repression of AP-1 activity through upregulation of ERK activity, which would result in a decrease in binding of AP-1 dimers containing c-Jun to the promoters of AP-1 responsive genes. However, two lines of evidence argue against

this. First, the activity of ERK detected in *at*RA-resistant SK-OV3 cells appeared to be much higher than that detected in *at*RA-sensitive CA-OV3 cells, regardless of *at*RA treatment. Second, the selective inhibition of ERK activity with PD98059 produced a twofold increase in AP-1 activity in both control and *at*RA treated sensitive cells. This effect did not appear to be related to PD98059 dose. Rather, it seemed as if the mechanisms through which ERK and *at*RA modulate AP-1 activity in these cells are divergent.

Protein phosphorylation is regulated by a balance between kinase and phosphatase activities. For example, JNK activity is modulated by MAP kinase phosphatases (MAPKP) [Lee et al., 1999]. PP2A, a major serine/threonine phosphatase in eukaryotic cells, has been implicated in cell cycle regulation and the modulation of protein kinase signaling modules [Janssens and Goris, 2001]. Additionally, PP2A has been shown to repress AP-1 activity by dephosphorylation of c-Jun on Ser-63 [Black et al., 1991; Al-Murrani et al., 1999]. Recent studies from our laboratory, by Vuocolo et al. [2003], show an increase in protein levels and activity of the catalytic subunit of PP2A (PP2Ac) in *at*RA-sensitive ovarian carcinoma cells. Based on these findings, we tested whether the increase in PP2Ac protein levels and activity correlated with the repression of AP-1 activity elicited by *at*RA in these cells. Phosphatase activity was induced by about threefold in CA-OV3 cells following 24 h of *at*RA treatment, as assayed using a peptide substrate that is highly specific for PP2A. To further investigate the specificity of the induction of phosphatase activity, the PP2A-specific inhibitor, endothall, was used in the phosphatase activity assay. Endothall is a cantharidin derivative used in the formulation of pesticides that has been shown to interact with the 36 kDa catalytic subunit (C) and the 65 kDa regulatory subunit (A) of PP2A [Erdodi et al., 1995]. The addition of endothall partially reversed the *at*RA-mediated induction of phosphatase activity in CA-OV3 cells, suggesting that at least half of the induced phosphatase activity could be attributed to PP2A. The inhibition of serine/threonine phosphatases with okadaic acid in *at*RA treated CA-OV3 cells also partially restored phosphorylated c-Jun protein levels to those of the control cells. Inhibition of PP2A activity with the specific inhibitor endothall, restored phosphorylated c-Jun protein levels to those of control samples at a concentration of 10 μM, in *at*RA-treated CA-OV3 cells. Moreover, a concentration of 25 μM endothall increased phosphorylated c-Jun protein levels above those of control samples in *at*RA-treated CA-OV3 cells. Furthermore, c-Jun and PP2Ac associated in vivo in control and *at*RA-treated in CA-OV3 cells. Finally, it appears that the specific inhibition of PP2A activity with endothall was able to reverse the repression of AP-1 activity by *at*RA in a dose dependent manner. Similar effects on AP-1 activity were obtained using fostriecin, another specific inhibitor of PP2A. Our results are consistent with those of Al-Murrani et al. [1999] and Shanley et al. [2001] who also showed that modulation of PP2A activity affects AP-1 activity, presumably by upregulation of phosphorylation of c-Jun on Ser-63. It remains to be determined whether the inhibition of PP2Ac results in increased JNK activity, as reported by Shanley et al. [2001]. Future experiments will include gene silencing of PP2Ac to provide further evidence in support of our hypothesis.

To conclude, this study presents evidence suggesting that PP2A is involved in mediating *at*RA induced repression of AP-1 activity, consequently leading to *at*RA-induced growth suppression. Presumably cell cycle arrest occurs via a mechanism that involves dephosphorylation by PP2A of c-Jun N-terminal transactivation domain, therefore reducing the transcription of those AP-1 responsive genes which regulate G_0/G_1 transition. Additionally, the upregulation of JunB protein, a known negative regulator of AP-1 activity, after *at*RA treatment of CA-OV3 cells, may also be involved in the *at*RA-mediated repression of AP-1 activity.

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