Protein Phosphatase 2A Associates with Rb2/p130 and Mediates Retinoic Acid-induced Growth Suppression of Ovarian Carcinoma Cells*

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Levels of Rb2/p130 protein are increased 5–10-fold following all-trans-retinoic acid (ATRA) treatment of the retinoid-sensitive ovarian adenocarcinoma cell line CAOV3, but not the retinoid-resistant adenocarcinoma cell line SKOV3. We found that this increase in Rb2/p130 protein levels in ATRA-treated CAOV3 cells was the result of an increased protein stability. Moreover, Rb2/p130 exhibited a decreased ubiquitination following ATRA treatment. Because phosphorylation frequently mediates ubiquitination of proteins, we examined the serine/threonine phosphatase activity in our CAOV3 cells following ATRA treatment. A significant increase in Ser/Thr phosphatase activity was found, which correlated with a rise in the level of protein phosphatase 2A (PP2A) catalytic subunit-α. In addition, co-immunoprecipitation and glutathione S-transferase pull-down studies demonstrated that PP2A and Rb2/p130 associate. We have made use of a battery of Rb2/p130 mutants to determine the sites dephosphorylated in response to ATRA treatment of CAOV3 cells. Obligate CDK4 phosphorylation sites seemed most important to the stability of the protein and are among the candidate sites that are dephosphorylated by PP2A following ATRA treatment. Finally, using both small interfering RNA specific to the catalytic subunit of PP2A and a variant of the SKOV3 cell line that overexpresses PP2A, we have shown that modulation of PP2A protein levels correlates with the ability of ATRA to inhibit growth of ovarian carcinoma cells. Our data suggest that ATRA mediates growth inhibition by stabilizing Rb2/p130 via a mechanism that involves induction of PP2A, an enzyme that can potentially dephosphorylate Rb2/p130, thereby protecting it from degradation by the proteasome.

Ovarian cancer accounts for ~33% of all newly diagnosed gynecologic cancers and 55% of gynecologic cancer deaths (1–4). The relatively high mortality rate associated with ovarian carcinoma is attributed to the late stage at which these tumors are usually diagnosed. This is unfortunate for the patient, as the tumors may have spread beyond the ovary at the time of diagnosis. Taking this into account, it is clear that an effective late stage therapy for ovarian cancer is desirable.

Retinoids are a group of natural and synthetic derivatives of vitamin A. Retinoids have been shown to play an important role in cell growth and differentiation by binding to their cognate nuclear receptors, the retinoic acid receptors (RARs)† and retinoid X receptors (RXRs) (5, 6). These nuclear receptors function as ligand-activated transcription factors, which can then bind to their consensus sequences in DNA (RAREs) and elicit the downstream biological effects of retinoids (7). Our studies have focused on the CAOV3 cell line, which is sensitive to all-trans-retinoic acid (ATRA) treatment, and the SKOV3 cell line, which is resistant to ATRA treatment. ATRA has been shown previously to inhibit the growth of CAOV3 ovarian carcinoma cells in the G1 phase of the cell cycle (8–10). Interestingly, this is also the phase of the cell cycle that is controlled by the retinoblastoma (Rb) family of proteins, including Rb2/p130 (11).

The Rb2/p130 phosphoprotein is a member of the Rb family of tumor suppressors. The three members of this family share a high degree of homology both in composition and in method of action (12–15). These “pocket” proteins maintain order at the G1 checkpoint of the cell cycle through their ability to bind and sequester members of the E2F family of transcription factors (11, 12, 16–18), which control the expression of genes involved in cell cycle progression. The growth regulatory activities associated with Rb2/p130 are mediated by its phosphorylation state, as this is clearly cell cycle-regulated (19–21); and Rb2/p130 has been shown to be a substrate for cyclin-cyclin-dependent kinase (CDK) complexes. Rb2/p130 has three apparent phosphorylation states. These species can be distinguished by their migration on SDS-PAGE and are presumably produced by various phosphorylations of the many CDK sites on Rb2/p130. The two faster migrating forms are stable and characteristic of the G0 state of the cell cycle, whereas the third, slowest migrating form is the most hyperphosphorylated and targets the protein for degradation. Degradation of Rb2/p130 consequently leads to progression through the cell cycle. It is most likely that this third, hyperphosphorylated species results from phosphorylation of CDK4-specific sites, converting the protein from

† These work was supported in part by National Institutes of Health Grants DE 13139 and CA 64945 (to K. J. S.) and National Institutes of Health Training Grant 5T32AI0710 (to the Department of Microbiology and Immunology, Temple University School of Medicine and S. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: RARs, retinoic acid receptors; RXRs, retinoid X receptors; ATRA, all-trans-retinoic acid; Rb, retinoblastoma; CDK, cyclin-dependent kinase; PP, protein phosphatase; PP2A.Cα, protein phosphatase 2A catalytic subunit α; GST, glutathione S-transferase; RT, reverse transcription; siRNA, small interfering RNA; CLBL, clasto-lactacystin β-lactone.
form 1 to 3 and leading to its subsequent degradation. Although the phosphorylation of the Rb2/p130 protein has been extensively studied, the dephosphorylation of this protein is not yet well characterized. It is known, however, that certain serine/threonine phosphatases act to dephosphorylate the closely related protein Rb (22–24) and therefore activate its growth-suppressive functions.

In this work, we set out to determine the mechanism by which ATRA treatment leads to the accumulation of Rb2/p130 protein and subsequent growth arrest. Our results show that the increase in Rb2/p130 levels caused by ATRA treatment involves a reduction in ubiquitination and proteasomal degradation of Rb2/p130. We also show a corresponding increase in the activity and protein level of protein phosphatase (PP) 2A following ATRA treatment. We were subsequently able to show a specific interaction between Rb2/p130 and PP2A that increased upon ATRA treatment. This interaction is functional, as a PP2A-specific phosphatase activity could be immunoprecipitated with Rb2/p130. We also show that inhibition of PP2A phosphatase activity by chemical inhibitors resulted in hyper-phosphorylation of Rb2/p130. This result supports the notion that PP2A dephosphorylates Rb2/p130 in CAOV3 cells. We also demonstrate that modulation of PP2A levels resulted in a corresponding change in the ability of ATRA to inhibit CAOV3 growth, suggesting that PP2A up-regulation is an important factor in the growth inhibition of CAOV3 cells mediated by ATRA. Taken together, our results suggest that PP2A is a mediator of retinoid-induced growth suppression in ovarian carcinoma cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The human ovarian adenocarcinoma cell lines CAOV3 and SKOV3 were obtained from American Type Culture Collection (Manassas, VA). SKOV3 cells overexpressing RARs and RXRs (SK-RAR/RXR cells) were previously described by our laboratory (25). All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 100 units/ml penicillin/streptomycin. Cells were maintained at all times in a 5% CO2 atmosphere at 37°C. ATRA treatments were performed on days 0, 2, and 4, and all cells were harvested on day 5 for preparation of lysates or cell counting. This was done to avoid any inaccuracies arising from changes in cell density. Phosphatase inhibitors were added to the medium at the indicated concentrations along with ATRA at 2-day intervals. A Phosphatase treatment of cell lysates was performed for 15 min at 37°C in assay buffer consisting of 50 mM Tris-HCl, 0.1 mM Na2EDTA, 5 mM dithiothreitol, 0.01% Brij 35, and 2 mM MnCl2 at pH 7.5.

Antibodies and Reagents—Anti-Rb2/p130 antibody (sc-317) was purchased from Santa Cruz Biotechnology. This antibody can recognize all phosphorylated forms of Rb2. Antibodies used against PP2A catalytic subunit- (sc-6110) is not able to immunoprecipitate the protein. Anti-hemagglutinin (sc-805) and anti-PPI (sc-7482) antibodies were purchased from Santa Cruz Biotechnology. A-Phosphatase (p0753s) was purchased from New England Biolabs Inc. A-Phosphatase is a manganese-dependent protein phosphatase with activity for phosphorylated serine, threonine, and tyrosine residues. The phosphatase inhibitors fostriecin, endothal, and okadaic acid were purchased from Sigma and were dissolved in phosphate-buffered saline. 10 mM okadaic acid will inhibit the activity of PP2A specifically, whereas 1 mM okadaic acid will inhibit the activities of both PP1 and PP2A. Both fostriecin and endothal are PP2C specific. ATRA was obtained as a generous gift from Hoffmann-La Roche.

Immunoprecipitation and Western Blotting—Whole cell lysates were prepared as described previously (32). Protein content of the lysates was determined by the Bradford assay (Bio-Rad). 100 μg of whole cell lysate was subsequently immunoprecipitated with 2 μg of antibody. Protein A/G PLUS-agarose beads (IP-04) purchased from Oncogene Research Products and were used to collect the antibody-antigen complexes. Immunoprecipitates were loaded on either a 7% or a 12% polyacrylamide gel and electrophoresed overnight. Gels were then transferred to polyvinylidene difluoro membranes by Western blotting. After blocking in 5% nonfat dry milk, the membranes were incubated with a 1:500 dilution of primary antibody. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) was used at a dilution of 1:4000, and immunoreactive proteins were visualized with the ECL™ enhanced chemiluminescence detection system (Amersham Biosciences).

Glutathione S-Transferase (GST) Pull-down Assay—Plasmids containing inserts corresponding to GST, GST-NH2, (amino acids 1–116 of Rb2/p130), GST-pocket (amino acids 417–1026 of Rb2/p130), and GST-COOH (amino acids 1027–1139 of Rb2/p130) were a generous gift of Dr. Antonio Giordano (Temple University). Fusion proteins were expressed in Escherichia coli BL21 cells and purified with glutathione-Sepharose beads. Purified GST fusion proteins were incubated with 500 μg of bacterial extract from CAOV3 cells treated with 10−6 M ATRA for 3 days. Incubation was carried out at 4°C overnight in binding buffer (60 mM NaCl, 1 μM EDTA, 20 mM Tris-HCl, 0.05% Nonidet P-40, 6 mM MgCl2, and 8% glycerol). The beads were then washed five times with wash buffer (500 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.05% Nonidet P-40, 6 mM MgCl2, and 8% glycerol). The bound proteins were eluted with SDS sample buffer, loaded onto a 10% polyacrylamide gel, and detected by Western blotting.

Phosphatase Assays—A radioactive protein serine/threonine phosphatase assay system was purchased from New England Biolabs Inc. Myelin basic protein substrate was prepared by phosphorylation of serine and threonine residues with [γ-32P]ATP (3000 Ci/mmol; Perkin-Elmer Life Sciences). A radioactive protein phosphatase assay system purchased from Promega (Madison, WI). The substrate for dephosphorylation in this assay, pRKATVB, is preferentially dephosphorylated by PP2A. It is known to be a poor substrate for PP1. Proteins were immunoprecipitated overnight with the corresponding antibodies and then incubated for 4 h with protein A/G-agarose beads. The beads were washed twice with immunoprecipitation wash buffer and twice with PP2A immunoprecipitation buffer (as described in the kit) and used in the assay with or without inhibitors.

Rb2/p130 Half-life Assays—The plasmids used in the preparation of in vitro transcribed and translated as well as in vivo wild-type and mutant Rb2/p130 proteins (pcDNA-HARb2 mutants) have been described previously (26). To determine the persistence of the Rb2/p130 phosphorylation mutants in vitro, the mutant constructs were first transcribed and translated in reticulocyte lysate. The TnT-Quick™ coupled transcription/translation system was purchased from Promega and used to make in vitro Rb2/p130. Plasmid DNA encoding the protein of interest was incubated with the rabbit reticulocyte lysate provided and [35S]methionine, [3H]cAMP-dependent protein kinase. After a 60-min incubation at 30°C, 2 μl of the protein lysate was used in degradation assays. In vitro transcribed and translated wild-type and mutant Rb2/p130 proteins in rabbit reticulocyte lysate were incubated with CAOV3 cell lysates for the indicated times at 37°C. An ATP-regenerating buffer consisting of 10 mM creatine phosphate, 2 mM ATP, and 100 μg/ml creatine phosphokinase was also added to the reactions. At the indicated time points, samples were collected and loaded onto a acrylamide gel, which was then dried in a gel dryer. The resulting bands were visualized using a PhosphorImager and quantitated using Opti-Quant™ software (Packard Instrument Co.).

To determine the persistence of these proteins in cells, the mutant Rb2/p130 plasmids were transiently transfected into CAOV3 cells using the calcium phosphate method. A cytomegalovirus-luciferase construct was cotransfected in all cases to correct for variation in transfection efficiency. 48 h after transfection, the medium was changed, and the cells were treated with 10 μg/ml cycloheximide. Cells were harvested at the indicated time points after cycloheximide treatment, lysed, and subjected to Western blot analysis. Western blotting was performed using the Total Lab™ software package from Nonlinear Dynamics (Newcastle Upon Tyne, United Kingdom).

Reverse Transcription (RT)-PCR Analysis—PP2Ac primers were purchased from Ranson Hill (Toronto, Canada). Primers were synthesized as follows: forward, 5′-CATCTGAATGGCTGGAAGT-3′; and reverse, 5′-TCACACATTTCCTTTCCCACTG-3′.PCR was performed using a 100 μg/ml of cDNA from CAOV3 cells following reverse transcription using the Total Lab™ software package from Nonlinear Dynamics (Newcastle Upon Tyne, United Kingdom).
product was sampled at three separate cycles to account for the linearity of the assay. Samples were run on a 10% polyacrylamide gel, analyzed using a PhosphorImager, and quantitated using Opti-Quant™. Samples were run on a 10% polyacrylamide gel, analyzed using a PhosphorImager, and quantitated using Opti-Quant™.

**RESULTS**

**ATRA Induces an Increase in Rb2/p130 Protein Levels in CAOV3 Ovarian Carcinoma Cells**—As shown in Fig. 1A, ATRA treatment of CAOV3 cells resulted in a significant increase in Rb2/p130 protein levels, although the mRNA levels remained relatively constant (Fig. 1B) (26). Because we have previously demonstrated that this increase is not seen in the ATRA-resistant cell line SKOV3 (26), we reasoned that understanding how ATRA treatment leads to the accumulation of Rb2/p130 could provide insight into the mechanism of retinoid-mediated growth inhibition of CAOV3 ovarian carcinoma cells.

**Rb2/p130 Protein Increase following ATRA Treatment Is Due to Increased Protein Stability**—Fig. 2 shows the turnover of the Rb2/p130 protein in CAOV3 cells following treatment with ATRA and cycloheximide. There was a clear increase in the stability of Rb2/p130 in ATRA-treated cells compared with ethanol-treated cells. Fig. 2B shows that ATRA treatment increased the half-life of the protein from 12 to >40 h. This experiment, along with the data in Fig. 1B, suggests that the increases in Rb2/p130 levels following ATRA treatment result from post-transcriptional or post-translational modifications.

**Ubiquitination of Rb2/p130 Decreases following ATRA Treatment in CAOV3 Cells**—To ascertain the mechanism responsi-
some inhibitor clasto-lactacystin β-lactone (CLBL). This inhibitor is the active metabolite of the previously described proteasome inhibitor lactacystin and has been shown to be a specific inhibitor of the proteasome (27, 28). This result indicates that the proteasome is responsible for degradation of Rb2/p130 in CAOV3 cells and that this system could possibly be modified by ATRA treatment. We next wanted to determine the extent of ubiquitination of the Rb2/p130 protein following ATRA treatment of CAOV3 cells. After treating CAOV3 cells with CLBL alone or with ATRA and CLBL, samples were immunoprecipitated with anti-Rb2/p130 antibody. The subsequent Western blot was then probed with an antibody to ubiquitin to assess the amount of ubiquitinated Rb2/p130. As shown in Fig. 3B, there was a clear decrease in the amount of ubiquitinated Rb2/p130 following ATRA treatment.

To rule out the possibility that the decreased degradation of Rb2/p130 resulted from ATRA-mediated changes in the levels of proteasome component proteins, Western blotting was performed to assay the levels of a variety of proteasome component proteins (HN3, LMP2, and XAPC7) following ATRA treatment. The representative Western blots shown in Fig. 3C demonstrate that the levels of these proteasome components did not change in response to ATRA treatment of CAOV3 cells. This suggests that the decreased degradation of Rb2/p130 following ATRA treatment of CAOV3 cells involves decreased targeting of ubiquitin to the protein, and not an alteration by ATRA of the expression of proteasome components.

**Increases in Phosphatase Activity in CAOV3 Cells Are Due to PP2A**—It has been shown that phosphorylation or dephosphorylation of target proteins can play a major role in whether or not they are ubiquitinated and degraded by the proteasome (29–31). Our laboratory has previously found that ATRA treatment causes both a decrease in hyperphosphorylated Rb2/p130 in CAOV3 cells and an increase in the amount of faster migrating Rb2/p130 forms, indicating that ATRA treatment results in a partial dephosphorylation of Rb2/p130 (32). Taking these data into account, we next performed a phosphatase assay specific for serine/threonine phosphatases that could dephosphorylate the Rb2/p130 protein and cause its increased persist-
Okadaic acid. Okadaic acid was added to the lysate and used at a concentration known to inhibit PP2A (10 nM) and at a higher concentration known to inhibit both PP2A and PP1 (1 μM) (33). Fosfocin was also used in the assay and is 40,000 times more specific for PP2A than any other serine/threonine phosphatase (34). Fig. 4A shows that both okadaic acid (at the low concentration) and fosfocin were able to almost completely inhibit the increase in phosphatase activity seen in CAOV3 lysates treated with ATRA. These data suggest that PP2A may be the major contributor to the up-regulation of phosphatase activity observed following ATRA treatment of CAOV3 cells. Taken together, these results implicate PP2A as a mediator of the previously shown ATRA-induced hypophosphorylation of Rb2/p130.

It has been shown previously that certain serine/threonine phosphatases can dephosphorylate members of the Rb family (29). Thus, along with our previous experiments, led us to investigate the expression of two major serine/threonine phosphatases, PP1 and PP2A, in CAOV3 cells following ATRA treatment. The PP2ACα protein was found to increase after 1 and 3 days of ATRA treatment by Western blotting (Fig. 4B). It is also interesting to note that the ATRA-resistant cell line SKOV3 expressed significantly lower levels of PP2A, although a small increase was observed after 3 and 5 days of ATRA treatment. This not only further implicates PP2A in the dephosphorylation of the Rb2/p130 protein in response to ATRA treatment, but may also give insight into the resistance to ATRA observed in SKOV3 cells.

We next performed an experiment to determine whether the increase in PP2ACα protein levels following ATRA treatment resulted from an increase in the amount of PP2ACα mRNA. 35S-labeled RT-PCR was performed on control and 3-day ATRA-treated CAOV3 cells. Samples were taken at three separate cycles to ensure that the results were in the linear range of the assay. It is clear from Fig. 4C that there was an up-regulation of PP2ACα mRNA in CAOV3 cells in response to ATRA treatment.

**Rb2/p130 Phosphorylation State Can Be Modulated by PP2A-specific Phosphatase Activity**—To provide evidence that PP2A phosphatase activity is directed toward Rb2/p130 following ATRA treatment, we chose to use the phosphatase inhibitors okadaic acid and endothal. Both okadaic acid and endothal are PP2A-specific inhibitors in vivo, though okadaic acid will inhibit PP1 at concentrations approaching 1 μM (33, 35). CAOV3 ovarian carcinoma cells were treated with ATRA and either endothal or okadaic acid for 24 h. As we have shown previously (32), Rb2/p130 extracted from ATRA-treated cells exhibited a partial dephosphorylation indicated by a faster migrating band (Fig. 5A). Cells that were treated with increasing concentrations of either okadaic acid or endothal exhibited a marked decrease in the migration of the Rb2/p130 band. This indicates that hyperphosphorylation of Rb2/p130 was induced as the result of inhibition of a specific phosphatase activity by okadaic acid and endothal. To further prove that the decrease in migration seen in Fig. 5A resulted from increased phosphorylation of Rb2/p130, we performed a similar assay using λ-phosphatase (Fig. 5B). Treatment of lysates with this phosphatase resulted in a total dephosphorylation of Rb2/p130, which then migrated faster than all other partially phosphorylated species.

**PP2A and Rb2/p130 Interact in Ovarian Carcinoma Cells**—To further investigate our hypothesis that Rb2/p130 is dephosphorylated by PP2A in response to ATRA treatment, we immunoprecipitated PP2ACα from CAOV3 cell lysates that had been treated with ATRA for 1, 3, and 5 days. We then performed SDS-PAGE and Western blotting using anti-Rb2/p130 antibody. The results shown in Fig. 6A show that PP2ACα and Rb2/p130 could be co-immunoprecipitated from CAOV3 cell extracts. Also, the lack of appreciable interaction in the ATRA-resistant cell line SKOV3 shown in Fig. 6A should be noted and was perhaps due to the low levels of PP2ACα protein in these cells. Fig. 6B shows the results of a GST pull-down assay, demonstrating that the COOH-terminal portion of Rb2/p130 could pull down PP2ACα from CAOV3 cell extracts. In addition, the amino-terminal and pocket regions of Rb2/p130, which are known to interact with CDK2, did not pull down PP2ACα. This indicates a specific interaction between the COOH terminus of Rb2/p130 and PP2A.

Fig. 6C shows the results from a nonradioactive phosphatase activity assay performed on CAOV3 cell lysates that were immunoprecipitated with either anti-Rb2/p130 or anti-PP2ACα. This phosphatase assay utilizes the substrate RRAPP7VA, which can be specifically dephosphorylated by PP2A, but is a poor substrate for the other major serine/threonine phosphatase, PP1. Immunoprecipitates generated from CAOV3 cell lysates using either anti-Rb2/p130 or anti-PP2ACα contained significant levels of serine/threonine phosphatase activity (Fig. 6C, first and fourth bars). Moreover, when this assay was repeated in the presence of the PP2A-specific inhibitors okadaic acid and endothal, 75% of the activity was lost (Fig. 6C, second, third, fifth, and sixth bars). These results show that immunoprecipitates generated using anti-Rb2/p130 antibody contained a PP2A-specific serine/threonine phosphatase activity, similar to immunoprecipitates generated with anti-PP2A antibody. These data indicate that the association of Rb2/p130 and PP2A as shown in Fig. 6 (A and B) is a functional interaction and support the assertion that PP2A dephosphorylates Rb2/p130 in response to ATRA treatment.

**Rb2/p130 Phosphorylation Mutants Are Differentially Degraded in Vitro and in CAOV3 Cells**—In light of our previous data implying that the phosphorylation state of Rb2/p130 is altered following ATRA treatment of CAOV3 cells and that the
subsequent increase in Rb2/p130 protein levels plays a role in CAOV3 sensitivity to ATRA, we wanted to determine the residue(s) of Rb2/p130 whose phosphorylation status might be altered in response to ATRA treatment. To do this, we utilized a panel of Rb2/p130 mutants that are phosphorylation-deficient at one or more proposed CDK4 phosphorylation sites due to mutation to alanine. Phosphorylation of CDK4 sites has been shown to be necessary for shifting of the Rb2/p130 protein from forms 1 and 2 to form 3, which is actively degraded by the proteasome (11, 13). If ATRA causes an increase in the Rb2/p130 protein level in CAOV3 cells by modulating its phosphorylation, it would be logical that at least one of these sites would be involved. Three CDK4 mutations were studied: T401A, S672A, and S1035A. A double mutant with both mutations at positions 401 and 672 was also used, as well as a CDK4 mutant containing all three sites mutated to alanine. As shown in Fig. 7A and Table II, in vitro transcription and translated Rb2/p130 was degraded when incubated with CAOV3 cell lysate. In contrast to the wild-type protein, mutants containing CDK4 phosphorylation sites mutated to alanine were degraded more slowly and exhibited a turnover rate more similar to that of wild-type Rb2/p130 incubated with ATRA-treated CAOV3 cell lysates. These results signify the importance of these residues in the degradation of the Rb2/p130 protein. These data are consistent with ATRA causing a decrease in the phosphorylation of Rb2/p130 and with the fact that only the hyperphosphorylated forms of Rb2/p130 are degraded by the proteasome.

in Fig. 7A and Table II, in vitro degradation analysis of various Rb2/p130 phosphorylation mutants. Representative results are shown for Rb2/p130 mutants translated in the presence of [35S] and subjected to the in vitro degradation assay as described under “Experimental Procedures.” Inhibitors were used at 10 nM and were added to a 96-well plate just before the immunoprecipitate was added and the assay was begun.
treated with cycloheximide, and samples were taken at various time points for analysis. We found that mutants containing alanine substitutions in CDK4 phosphorylation sites were significantly increased in stability compared with wild-type Rb2/p130, similar to the results from the in vitro experiment. When expressed in untreated CAOV3 cells, these mutant proteins mimicked the response seen with wild-type Rb2/p130 in the presence of ATRA (as shown in Fig. 7B). This indicates that CDK4 sites are also involved in the regulation of Rb2/p130 degradation in CAOV3 ovarian carcinoma cells and may be a target of ATRA-mediated growth inhibition through modulation by PP2A following ATRA treatment.

Extent of Growth Suppression by ATRA Correlates with Levels of PP2A and Rb2/p130—Our finding that PP2A could play a role in the suppression of growth by ATRA in ovarian carcinoma cells through its ability to increase Rb2/p130 levels. Previous work in our laboratory has shown that ATRA-resistant SKOV3 cells can be made sensitive to ATRA by overexpression of RARα and RXRα (SK-RAR/RXR cells) (25). CAOV3, SKOV3, and SK-RAR/RXR cells were treated with ATRA, and cell counts were performed at 1 and 5 days after treatment. Consistent with previous results from our laboratory (25), growth of the SK-RAR/RXR cell line was inhibited to an intermediate level (20–25%) after 5 days of ATRA treatment. In contrast, the parental SKOV3 cell line was completely resistant to the growth inhibitory affects of ATRA (Fig. 8A).

Whole cell lysates were then prepared from control and ATRA-treated cells and subjected to SDS-PAGE and Western blotting for Rb2/p130 and PP2A. Fig. 8B shows the previously described increase in PP2A levels following ATRA treatment of CAOV3 cells, but not SKOV3 cells. Interestingly, ATRA treatment of the intermediately ATRA-sensitive SK-RAR/RXR cell line resulted in an intermediate increase in anti-PP2A antibody. Lanes marked with C are ethanol-treated controls. C, siRNAs against PP2A (PP2Ac) were synthesized using the Gene Silencer™ siRNA preparation kit in vitro siRNA synthesis kit available from Ambion. Sequences used to make in vitro siRNAs against PP2A (see Table I) were described previously (51). CAOV3 cells were treated with ATRA on day 1. On day 2, a combination of the three different siRNAs was transfected into these cells at a final concentration of 25 or 50 nM (in the plates) for 4 h in the presence of 10\(^{-6}\) M ATRA. 72 h after transfection (day 5), the cells were harvested, and Western blotting for PP2A was performed. Growth analysis of CAOV3 cells treated with siRNAs and ATRA is also shown (lower panel). Direct cell counting was performed 72 h after treatment with siRNA to assess the sensitivity of CAOV3 cells following knock-down of PP2A expression. The control is CAOV3 cells treated with ethanol for 72 h. *, statistical significance between 25 and 50 nM siRNA samples and the control via the paired t test (p < 0.05).

### Table II

**Half-lives of Rb2/p130 phosphorylation mutants in vitro and in CAOV3 cells**

The half-lives of Rb2/p130 mutants were determined from the data in fig. 7 Values represent the means ± S.D. of three independent experiments. WT, wild-type.

|        | In vitro |        | CAOV3 |
|--------|----------|--------|-------|
| Rb2/p130 |         |        |       |
| WT      | 4.0 ± 0.12 | WT    | 3.6 ± 0.26 |
| WT + ATRA | >8      | WT + ATRA | >24   |
| S672A   | 1.65 ± 0.4  | S672A  | 5.2 ± 0.46 |
| T401A   | 4.2 ± 0.26  | T401A  | 12.2 ± 0.34 |
| T401A/S672A | 6.2 ± 0.23 | T401A/S672A | 8.1 ± 0.32 |
| S1035A  | ≥8       | S1035A | 17.7 ± 0.22 |
| CDRK4   | >8       | CDRK4  | >24   |

**Fig. 8.** Analysis of the correlation of Rb2/p130 and PP2A levels with extent of growth suppression mediated by ATRA treatment of ovarian carcinoma cells. A, shown is the growth of ovarian carcinoma cells treated with 10\(^{-6}\) M ATRA. ATRA-resistant SKOV3 cells (SK), ATRA-sensitive CAOV3 cells (CA), and partially ATRA-sensitive SK-RAR/RXR cells were treated with ATRA. Direct cell counting was performed at days 1 and 5 to determine the growth inhibition of the cell lines. B, Western blot analysis was carried out with PP2A and Rb2/p130 in ovarian carcinoma cells treated with ATRA. Whole cell lysates of ovarian carcinoma cells treated with 10\(^{-6}\) M ATRA for 1, 3, or 5 days were subjected to Western blot analysis using anti-PP2A antibody. Lanes marked with C are ethanol-treated controls. C, siRNAs against PP2A (PP2Ac) were synthesized using the Gene Silencer™ siRNA preparation kit in vitro siRNA synthesis kit available from Ambion. Sequences used to make in vitro siRNAs against PP2A (see Table I) were described previously (51). CAOV3 cells were treated with ATRA on day 1. On day 2, a combination of the three different siRNAs was transfected into these cells at a final concentration of 25 or 50 nM (in the plates) for 4 h in the presence of 10\(^{-6}\) M ATRA. 72 h after transfection (day 5), the cells were harvested, and Western blotting for PP2A was performed. Growth analysis of CAOV3 cells treated with siRNAs and ATRA is also shown (lower panel). Direct cell counting was performed 72 h after treatment with siRNA to assess the sensitivity of CAOV3 cells following knock-down of PP2A expression. The control is CAOV3 cells treated with ethanol for 72 h. *, statistical significance between 25 and 50 nM siRNA samples and the control via the paired t test (p < 0.05).
PP2A\(\alpha\) levels compared with CAOV3 cells. Thus, the PP2A\(\alpha\) level correlates with growth inhibition mediated by ATRA in ovarian carcinoma cells. However, PP1 showed no increase in protein levels in any of the three cell lines tested when treated with ATRA.

As shown in Fig. 8C, a combination of three siRNAs directed against PP2A\(\alpha\) resulted in an ~75% reduction in the level of PP2A protein induced following ATRA treatment. As expected, this gene silencing also resulted in a reduction in Rb\(2/p130\) levels. Prevention of the induction of PP2A\(\alpha\) and the accumulation of Rb\(2/p130\) significantly altered the ability of ATRA to inhibit CAOV3 cell growth. Growth of CAOV3 cells treated with ATRA alone was inhibited 40–45% by day 4 following treatment. However, when these cells were treated with ATRA and a combination of the three PP2A\(\alpha\) siRNAs, they exhibited only a 20% inhibition of growth after 72 h of siRNA treatment. Thus, prevention of the ATRA-dependent induction of PP2A\(\alpha\) expression leads to a reduction in the level of Rb\(2/p130\) and significantly reduces the extent of growth suppression observed following ATRA treatment of CAOV3 cells.

**DISCUSSION**

In this work, we investigated the mechanisms responsible for the substantial increase in Rb\(2/p130\) protein levels that contribute to growth inhibition in ATRA-treated CAOV3 cells. Our results show that 1) the Rb\(2/p130\) protein has an increased stability in ATRA-treated cells; 2) this increased stability correlates with a decreased ubiquitination of the Rb\(2/p130\) protein in ATRA-treated cells; 3) there is an increased activity/amount of serine/threonine phosphatase PP2A\(\alpha\) following ATRA treatment; 4) Rb\(2/p130\) associates with PP2A and can be immunoprecipitated with PP2A-specific protein phosphatase activity; 5) CDK4 phosphorylation sites are likely targets for dephosphorylation by PP2A in CAOV3 cells; and 6) the expression of both PP2A\(\alpha\) and Rb\(2/p130\) correlates with ATRA-dependent growth inhibition of CAOV3 ovarian carcinoma cells.

We have shown that Rb\(2/p130\) becomes less ubiquitinated following ATRA treatment. Recently, other laboratories have shown that ATRA affects the proteasome-dependent degradation of other cell cycle proteins. Borriello et al. (36) have shown that ATRA treatment of neuroblastoma cells causes an increase in the CDK inhibitor protein p\(\gamma\)\(^2\)\(^\text{Rbp1}\) through a mechanism involving decreased proteasomal degradation. On the other hand, CDK4 has been shown to be degraded more rapidly following treatment with ATRA in human bronchial epithelial cells compared with controls (37). Whether the effect is an increase or decrease in proteasomal degradation, it is clear from our studies and the work of others that ATRA can have an effect on the ubiquitin/proteasome system.

Although Rb\(2/p130\) can inhibit cell growth through direct interaction and sequestration of E2F (18), it can also recruit histone deacetylase (38–41), which reduces transcriptional activity. Rb\(2/p130\) can also directly inhibit CDK activity through a conserved domain it shares with p\(107\), but not with Rb (42–46). These functions, as well as the degradation of Rb\(2/p130\), are presumably regulated by phosphorylation. The phosphorylation of Rb\(2/p130\) has recently been extensively investigated (20). Although most of the 22 apparent phosphorylation sites on Rb\(2/p130\) can be phosphorylated by both CDK2 and CDK4 (20), there are three sites that can apparently be phosphorylated only by CDK4. These are serine 672, threonine 401, and serine 1035 (20). Interestingly, CDK4 phosphorylation sites have previously been implicated in function in proteasomal degradation of Rb family proteins (21, 47). Less is known about the dephosphorylation of the Rb\(2/p130\) protein; however, previous results from this laboratory have demonstrated that Rb\(2/p130\) becomes hypophosphorylated following ATRA treatment (32). This hypophosphorylation of Rb\(2/p130\) in CAOV3 cells following ATRA treatment is also visible in Fig. 8B. It is also visible in the SK-RAR/RXR cell line, which expresses increased amounts of PP2A compared with SKOV3 cells.

Our data suggest that ATRA treatment leads to the partial hypophosphorylation of Rb\(2/p130\), which is caused by an increased level/activity of PP2A. We believe that the increased stability of Rb\(2/p130\) in response to ATRA treatment occurs as a result of its hypophosphorylation. Our data also suggest that it is this increased Rb\(2/p130\) protein stability that is, at least in part, responsible for the growth inhibition seen in CAOV3 cells following ATRA treatment. Studies reported by Yan and Mumba (23) showed that there are distinct roles for both PP1 and PP2A in the dephosphorylation of the Rb protein under normal conditions. Our results demonstrate that PP2A is a phosphatase exhibiting increased protein expression (catalytic subunit-\(\alpha\)) and activity in CAOV3 cells treated with ATRA. This was somewhat of a surprise in light of previous results by Tawara et al. (48), who showed that ATRA can inhibit the expression of the PP2A\(\alpha\) protein in HL-60 cells. Our experiments (Western blotting and PCR) show that PP2A\(\alpha\) protein and mRNA levels were increased in CAOV3 cells following ATRA treatment. We have also shown an association of the COOH-terminal region of Rb\(2/p130\) with PP2A\(\alpha\). It is important to note that it is not known whether this interaction is direct or whether it is mediated by one or more other proteins. Data confirming this interaction also proved that the interaction of Rb\(2/p130\) and PP2A is a functional one, as a PP2A-specific phosphatase activity could be immunoprecipitated along with Rb\(2/p130\).

Our studies with Rb\(2/p130\) CDK4 phosphorylation site alanine mutants provided information as to where PP2A may be acting to dephosphorylate and therefore stabilize Rb\(2/p130\). CDK4 sites such as serine 1035, threonine 401, and serine 672 are likely among the sites altered by PP2A in response to ATRA, as these mutant proteins, when harvested from ethanol-treated control cells, exhibited a similar half-life compared with wild-type Rb\(2/p130\) from cells treated with ATRA. This implies that the lack of phosphorylated CDK4 sites of mutant Rb\(2/p130\) mimics the response seen with wild-type Rb\(2/p130\) in the presence of ATRA.

Previous experiments in our laboratory have shown that antisense directed against Rb\(2/p130\) mRNA expressed stably in CAOV3 cells renders these cells resistant to the growth inhibitory effects of ATRA. These data bring to light a correlation between the level of Rb\(2/p130\) and the extent of CAOV3 growth inhibition by ATRA. Fig. 8 further supports this point.

PP2A also appears to be required for the full growth inhibitory effect of ATRA. Using siRNA technology to reduce PP2A levels in CAOV3 cells resulted in decreased sensitivity of CAOV3 cells to the growth inhibitory effects of ATRA. Further evidence of the role of PP2A in ATRA-mediated growth inhibition is provided by a variant of the normally ATRA-resistant SKOV3 cell line that overexpresses RARs and RXRs. Using this cell line, we were able to show that ATRA caused an increase in expression of PP2A\(\alpha\) and that this increased expression correlated with growth suppression of ovarian carcinoma cells. Implicit in these results is the observation that increases in RARs and RXRs are able to affect an increase in the amount of PP2A\(\alpha\) protein. This is supported by the findings of Chen et al. (49), who have recently shown that expression of PP2A\(\alpha\) mRNA is regulated by retinoid receptors during development.

In this work, we have shown that the accumulation of the

\(^{2}\)K. J. Soprano, unpublished data.
Rb2/p130 protein seen following ATRA treatment of CAOV3 cells is due to a decreased ubiquitination. This decreased ubiquitination is most likely due to hypophosphorylation caused by an increase in the levels and activity of PP2A. We have demonstrated, for the first time in ovarian carcinoma, a specific interaction between PP2A and the COOH terminus of Rb2/p130. We have also shown that immunoprecipitated Rb2/p130 could pull down a PP2A-specific phosphatase activity, indicating that the association of Rb2/p130 and PP2A is a functional one. Furthermore, in Fig. 5, we have shown that specific inhibition of PP2A using two independent agents (okadaic acid and endothal) caused an increase in the phosphorylation of Rb2/p130 based on migration on SDS-polyacrylamide gels. This implies that PP2A dephosphorylates Rb2/p130 under normal conditions. It is also important to note the dephosphorylation of Rb2/p130 in the samples from just ATRA-treated cells in this experiment. The small change in migration in these samples indicates this fact. The actual dephosphorylation of Rb2/p130 that leads to its increased stability is subtle, as it most certainly does not involve a large number of sites. In conclusion, our study identifies PP2A as an indirect mediator of CAOV3 cell growth inhibition by ATRA through a mechanism involving increased stability of the Rb2/p130 protein.

Acknowledgments—We thank Hoffmann-La Roche for the retinoic acid used in this study. We also thank Dr. Antonio Giordano for the generous gift of the GST-Rb2/p130 constructs used in this work.

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