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Inhibition of PP2A, but not PP5, Mediates p53 Activation by Low Levels of Okadaic Acid in Rat Liver Epithelial Cells

Donald J. Messner,[1] Charles Romeo,[3] Alton Boynton,[4] and Sandra Rossie[1,2]*

[1]Department of Biochemistry, Purdue University, West Lafayette, Indiana
[2]Purdue Cancer Center, Purdue University, West Lafayette, Indiana
[3]Laboratory of Neurobiology, NIEHS, Research Triangle Park, North Carolina
[4]Northwest Biotherapeutics, Bothell, Washington

Abstract The microbial toxin okadaic acid (OA) specifically inhibits PPP-type ser/thr protein phosphatases. OA is an established tumor promoter with numerous cellular effects that include p53-mediated cell cycle arrest. In T51B rat liver epithelial cells, a model useful for tumor promotion studies, p53 activation is induced by tumor-promoting (low nanomolar) concentrations of OA. Two phosphatases sensitive to these concentrations of OA, PP2A and protein phosphatase 5 (PP5), have been implicated as negative regulators of p53. In this study we examined the respective roles of these phosphatases in p53 activation in non-neoplastic T51B cells. Increases in p53 activity were deduced from levels of p21 (cip1) and/or the rat orthologue of mdm2, two p53-regulated gene products whose induction was blocked by siRNA-mediated knockdown of p53. As observed with 10 nM OA, both phospho-ser15-p53 levels and p53 activity were increased by 10 μM fostriecin or SV40 small t-antigen. Both of these treatments selectively inhibit PP2A but not PP5. siRNA-mediated knockdown of PP2A, but not PP5, also increased p53 activity. Finally, adenoviral-mediated over-expression of an OA-resistant form of PP5 did not prevent increased phospho-ser15-p53, p53 protein, or p53 activity caused by 10 nM OA. Together these results indicate that PP5 blockade is not responsible for OA-induced p53 activation and G_1 arrest in T51B cells. In contrast, specific blockade of PP2A mimics p53-related responses to OA in T51B cells, suggesting that PP2A is the target for this response to OA. J. Cell. Biochem. 99: 241–255, 2006.

Key words: okadaic acid; ser/thr protein phosphatase; PP2A; PP5; p53; tumor promotion

The p53 tumor suppressor pathway plays a major role in orchestrating the cellular response to DNA damage in mammalian cells, and is defective in many human cancers [Giacca and Kastan, 1998; Zhou and Elledge, 2000; Liu and Kulesz-Martin, 2001]. In addition to genetic defects in p53 itself, the pathway can be compromised by alteration of downstream mediators of the p53 response, or in upstream p53 activators. The transcriptional activity of p53 is controlled by activity and levels of co-regulators as well as by the oligomeric state and post-translational modifications of the p53 protein itself, which vary with cell type and stimuli. Phosphorylation of p53 at serine and threonine residues is known to accompany its activation [Meek, 2004], however many details concerning how the phosphorylation status of p53 is controlled are still poorly understood [Bode and Dong, 2004].

The microbial toxin okadaic acid (OA) is a specific ser/thr phosphatase inhibitor that has been shown to be a potent tumor promoter in several animal and cell-based models [Bialojan and Takai, 1988; Suganuma et al., 1988; Nishiwaki-Matsushima et al., 1992; Suganuma et al., 1992; Messner et al., 2001]. By blocking ser/thr protein dephosphorylation, OA indirectly elevates phosphorylation and activation of

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Donald J. Messner's present address is Bastyr University, 14500 Juanita Drive, Kenmore, WA 98028.
E-mail: DMessner@bastyr.edu

*Correspondence to: Sandra Rossie, Department of Biochemistry, Purdue University, 175 S. University St., West Lafayette, IN 47907. E-mail: rossie@purdue.edu

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pathways that contribute to cell proliferation and may also override or bypass cell cycle checkpoints [Fernandez et al., 2002]. However, in many cell types OA increases p53 phosphorylation, the expression of p53 responsive gene products, and causes cell cycle arrest or apoptosis [Mordan et al., 1990; Zhang et al., 1994; Lohrum and Scheidtmann, 1996; Yan et al., 1997; Milczarek et al., 1999; Messner et al., 2001]. The basis for OA-induced p53 activation and G_1 arrest has not been clearly established, but several OA-sensitive ser/thr phosphatases have been implicated in the phosphoregulation of p53. Identifying the specific phosphatase(s) responsible for OA-induced p53-mediated G_1 cell cycle arrest is integral to understanding tumor promotion by OA and will clarify the role of ser/thr phosphatases in p53-mediated cell cycle control.

Ser/thr protein phosphatase 2A (PP2A, IC_{50} near 0.05 nM), and ser/thr protein phosphatase 1 (PP1, IC_{50} near 50 nM) account for the majority of OA-sensitive phosphatase activity in cells, and each is capable of dephosphorylating p53 in vitro [Scheidtmann et al., 1991; Takenaka et al., 1995]. In addition, inhibition of cellular PP2A activity by SV40 small t-antigen increased p53 phosphorylation and activity [Yan et al., 1997]. Protein phosphatase 5 (PP5) is typically less abundant, however its high sensitivity (IC_{50} near 1 nM) [Honkanen and Golden, 2002] makes it likely that PP5 mediates some of the cellular responses to OA previously attributed to PP2A. In this regard, recent gene knockdown studies have implicated PP5 as a negative regulator of p53 function. Antisense blockade of PP5 expression was shown to cause G_1 arrest in cells with functional p53, but not in cells with defective p53 or in p53 null cells [Zuo et al., 1998]. Cell growth inhibition was accompanied by increased p21 (cip1) mRNA levels, as well as increased p53 phosphorylation. Importantly, PP1, PP2A, and PP5 are all known to interact with p53 binding partners and kinases, and so may regulate p53 stability and activity indirectly [Helps et al., 1995; Okamoto et al., 2002; Ali et al., 2004].

Mis-regulation of p53 and normal cell cycle progression by OA treatment are very likely to be relevant to the process of tumor promotion by OA. Exactly how has been difficult to establish, in part because experimental models and treatment conditions used to study one aspect of OA action are often not well suited for studying another aspect. It is frequently not possible to compare responses to OA from different studies and cell lines, as they are dependent on numerous factors including cell type, dose, and time. Non-neoplastic epithelial T51B rat liver cells have been used extensively as a cell-based model to study tumor promotion [Swierenga et al., 1978; Boynton et al., 1984; Messner et al., 2001]. In a previous study we characterized the effect of low levels of OA on both cell cycle responses and tumor promotion in T51B cells, as measured by growth in soft agar [Messner et al., 2001]. This enabled us to minimize differences attributable to cell context or technical differences in experiments, and permitted us to compare the effect of OA on cell cycle responses with its tumor promotion potential in a non-neoplastic cell line. We found that T51B cells exhibited p53 activation and G_1 arrest during short-term treatment, as well as tumor promotion following long-term treatment, as measured by colony formation in soft agar. The onset of p53-related effects of OA occurred within the same low concentration range as that required for tumor promotion.

The goal of the present study was to identify the phosphatase target(s) responsible for OA-induced p53 activation and G_1 arrest in an experimental model relevant for tumor promotion. The low concentrations of OA required for p53 activation in our previous study effectively rule out a role for PP1 in the OA-induced activation of p53 [Honkanen and Golden, 2002], but cannot distinguish between PP2A and PP5 in cellular studies, due to the complex pharmacokinetic properties of OA that dictate its accumulation and compartmentation in cells [Janssens and Goris, 2001]. Previous studies on p53 activation in response to PP2A or PP5 downregulation have been performed using cell types incompatible with tumor promotion studies. Furthermore, no single model system has been used to examine the relative roles of both of these two phosphatases on p53 regulation. To address these various deficiencies, we employed a combination of approaches that selectively affect either PP2A or PP5 to determine whether blockade of one or both of these enzymes play a role in p53 activation in T51B cells. These included: (i) pharmacologic treatment with fostriecin, a drug that inhibits PP2A at levels 10,000-fold less than required for PP5 inhibition [Honkanen and Golden, 2002], (ii) expression of small t-antigen, which blocks normal PP2A

function [Janssens and Goris, 2001], (iii) siRNA knockdown experiments, and (iv) OA treatment of cells expressing an OA-resistant form of PP5. The results of these experiments indicate that blockade of PP5 is not required for p53 activation by low, tumor promoting doses of OA in T51B cells, and are consistent with a specific role for PP2A in this process.

MATERIALS AND METHODS

Materials

The sodium salt of OA was purchased from Alexis Biochemicals (San Diego, CA). Fostriecin was obtained from the National Cancer Institute. The origins of antibodies, cell culture reagents, and specialty reagents are noted in the appropriate Materials and Methods section. Other reagents were from standard suppliers.

Cell Culture and Biochemical Analyses

T51B cells were maintained in Eagle's basal media (Gibco) supplemented with 10% newborn calf serum (Atlanta Biologicals), 2 mM L-glutamine, and penicillin/streptomycin (Gibco). They were grown at 37°C and 5% CO_2 and used within 10 passages from the initial stock of cells. In general, cells were near or at confluence at the time of harvest, accomplished by first rinsing on ice in PBS supplemented with phosphatase and protease inhibitors (0.1 mg/ml PMSF, 1 mM sodium orthovanadate, 10 μg/ml benzamidine, and 1 μg/ml each pepstatin A, chymostatin, and leupeptin) [Messner et al., 2001]. Two cell harvesting and analysis protocols were used as follows.

For samples analyzed by SDS–PAGE and Western blot only, cells were scraped in 2% SDS, TBS (25 mM Tris-HCl, 120 mM NaCl, pH 7.5), 2 μM Zn acetate with phosphatase and protease inhibitors and immediately boiled. Protein concentration was determined using a modified Lowry assay and equal amounts of protein (Figs. 2, 3, and 6) or equal volume aliquots (Fig. 1) were subjected to SDS–PAGE as described [Messner et al., 2001]. Following transfer to PVDF membranes (BioRad Laboratories) samples were probed with antibodies according to manufacturers' instructions. Commercially available antibodies and their sources were: total p53, cyclin E, mdm2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (M-19 and R-19, M-20, SMP14, and FL-335, respectively; Santa Cruz Biotechnology, Santa Cruz, CA); phospho-ser15-p53 and phospho-thr180-tyr182-p38 (Cell Signaling, Beverly, MA); p21 (cip1), PP2A, and PP4 (Upstate Biotechnology, Lake Placid, NY); cyclin A (Oncogene Research Products, Cambridge, MA); PP5 monoclonal (Transduction Laboratories, now BD Biosciences, CA). PP5 antiserum generated using a 15 amino acid peptide corresponding to a region of PP5 between the TPR and catalytic domains [Bahl et al., 2001], was used for immunoprecipitation, for Western blots shown in Figures 2, 3, and 6, and for immunofluorescence studies. All Western blots were performed using secondary antibodies conjugated with horseradish peroxidase (Jackson Immunoresearch, West Grove, PA) and the ECL-plus system (Amersham, Arlington Heights, IL). Quantitative evaluation of data similar to that shown in Figures 2, 3, and 6 was performed using the Odyssey infrared imaging system (Licor Biosciences, Omaha, NE). Secondary antibodies for the Odyssey were conjugated with IR-680 dye (Molecular Probes, Eugene, OR) or IR-800 dye (Rockland, Gilbertson, PA).

Samples used for T51B cell phosphatase activity measurements (Figs. 4B and 5B) were harvested in Cell IP buffer (5 mM HEPES, 40 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% triton X-100, 1% glycerol, 5 mM NaF, pH 7.4) supplemented with phosphatase and protease inhibitors. Parallel analyses of recombinant flag-PP5 started with the purified enzyme diluted in Cell IP buffer. Samples were supplemented with 0.1 mg/ml BSA, incubated on ice for 30 min, centrifuged at $13{,}000 \times g$ for 30 min, and the solubilized material was immunoprecipitated with PP5 antiserum and protein A Sepharose. The immune pellets were washed sequentially with Cell IP buffer containing BSA and protease inhibitors, Cell IP buffer without BSA, and finally with phosphatase assay buffer B (50 mM Tris, 1 mM EDTA, 1 mM EGTA, pH 7.6). The beads were resuspended in phosphatase assay buffer A (see below) and used directly for Western blot or PNPP phosphatase assay.

Generation of Flag-PP5-Y451A and Related Reagents

For the pCI-flag vector, a pair of overlapping adaptor oligonucleotides containing the flag epitope and a short linker peptide sequence were synthesized and inserted into the *Eco*R1

and *Mlu*I sites within the multicloning site of pCI (Promega). For pCI-flag-PP5, rat PP5 (RNPPT, GenBank Accession X77237) was cloned by PCR as an *Mlu*I–*Not*I fragment and inserted into the pCI-flag vector. The forward PCR oligo was derived from nt 13–33 of the RNPPT sequence and also contained an *Mlu*I site and a GC clamp at the 5′ end (cgcgggac cgt-ATGGCGATGGCGGAGGGCGAG). The reverse PCR oligo was derived from nt 1,509–1,489 and contained a *Not*I site and GC clamp (cgcggg-gcggccgctttCATCATTCCTAGCTGCAGCAG). Flag-PP5 expressed in mammalian cells from pCI has the sequence MVDYKDDDDKLGG-GATR-PP5 (flag underlined). For PP5-Y451A, a reverse oligo that introduces both the Y451A mutation and a silent *Hind*III site was used with a PP5 forward oligo overlapping the *Nsi*I site at nt 1,067 to generate an *Nsi*I–*Hind*III PCR product. A silent *Hind*III forward oligo and the PP5 *Not*I reverse oligo were used to generate a *Hind*III–*Not*I product. The pCI-flag-PP5 construct was cut with *Nsi*I and *Not*I, gel purified, and used as the acceptor for both *Nsi*I–*Hind*III (Y451A) and *Hind*III–*Not*I fragments in a three-part ligation. Both flag-PP5 and flag-PP5-Y451A were sequenced to confirm their integrity.

The adenoviral expression vector was constructed by cloning the *Xho*I/*Not*I fragment of pCI-flag-PP5-Y451A into the pAd5CMVK-NpA shuttle vector (University of Iowa, Iowa City, IA). This construct was co-transfected with an adenovirus backbone in HEK293 cells and the recombinant virus amplified, purified, and titered by the Gene Transfer Vector Core at the University of Iowa [Anderson et al., 2000]. The purified virus (AdV-flag-PP5-Y451A) was aliquoted and stored at −80°C and subjected to no more than three freeze/thaw cycles prior to use. Control virus without the PP5 insert was treated in the same way.

Over-Expression and Knockdown Techniques

Transfection of T51B cells with pCI-flag-PP5-Y451A was performed with Fugene (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's recommendations. The cells were harvested 24 h later and analyzed for PP5 activity and protein. Infection of T51B cells with AdV-flag-PP5-Y451A was accomplished by adding either crude lysate (Fig. 4B) or purified viral particles (Figs. 5–7) to confluent cells for 12–16 h. The moi, (multiplicity of infection, or plaque forming units of virus added per cell), was calculated by counting cells from a parallel plate and using virus concentrations provided by the production service. The virus containing media was then replaced with fresh complete media and expression was allowed to proceed for 1–5 days. Where indicated, OA was added after the adenovirus was removed, during the end of the expression phase. Adenovirus Ad-t, expressing the SV40 small t-antigen behind the CMV promoter [Porras et al., 1996], was obtained from Dr. Kathleen Rundell, Northwestern University. Infection of T51B cells was performed at moi = 6 as described above for AdV-flag-PP5-Y451A, using culture media containing either Ad-t or a control virus that included the CMV promoter but expressed no protein (AdCMV). Fresh media was added 24 h after the start of infection, and the cells harvested for Western blot analysis 40 h later.

Transfection with siRNA was performed with lipofectamine 2000 (Invitrogen Corp., Carlsbad CA). For p53 knockdown, siRNA designed for rat p53 was purchased from Ambion (Austin, TX) as was the negative control siRNA. For PP2A knockdown, a mixed pool of 4 RNA 21mers designed to be selective for the alpha isoform of the catalytic subunit of human PP2A was purchased from UBI (Lake Placid, NY). For PP5 knockdown, two distinct RNAs were synthesized using an siRNA construction kit (Ambion) from synthetic DNA oligos: PP5-r13 (AAGAAGTACATCAAAGGTTAC) and PP5-r68 (AATTATTGTGACCAGATGGGA). The amount of RNA needed to achieve the indicated final concentration was combined with a fourfold excess of lipofectamine 2000 in serum-free Optimem I media (Gibco) at concentrations similar to those recommended by Invitrogen. Subconfluent cells in Optimem I were exposed to RNA-lipofectamine for 4 h, and then the cells were returned to normal complete media for 3–4 days. Where noted OA treatment occurred during the final 48 h.

Generation of Recombinant Flag-PP5 and PP5 Phosphatase Assays

Flag-PP5 was cloned into pET-21a with *Bam*HI/*Eco*RI as described for PP5 alone and the GST fusion protein expressed, purified, and cleaved as described [Skinner et al., 1997]. The resulting bacterially expressed and thrombin-cleaved protein had the sequence GSGSEFPMVDYKDDDDKLGGGATR-PP5

(flag underlined). The OA sensitivity of recombinant flag-PP5-WT and flag-PP5-Y451A (Fig. 4A) were analyzed as previously described [Skinner et al., 1997], using ^{32}P-casein as substrate.

The phosphatase assay used for measuring PP5 in T51B cell extracts (Figs. 4B and 5B) was developed using recombinant flag-PP5 and modification of conditions used by Takai and Mieskes [1991]. Following immunoprecipitation with PP5 antiserum, aliquots of the immune pellets were suspended in phosphatase assay buffer A (50 mM Tris, 4 mM $MnCl_2$, 4 mM $MgCl_2$, 1 mM EGTA, 0.1% 2-mercaptoethanol, pH 7.6) and pre-incubated with or without OA at 30°C for 5 min. PNPP (para-nitrophenyl phosphate) was added to a final concentration of 200 mM and the reaction was allowed to proceed for 30 min–2 h. The reaction was quenched by addition of 20 volumes 0.25 M NaOH and centrifuged for 10 min at 13,000×*g*. Absorbance was measured at 410 nm and enzyme activity was calculated using the extinction coefficient for PNP of 17,800 AU/nmol. The assay was performed in duplicate. Under these conditions, recombinant flag-PP5 in the absence of antibody displayed a specific activity approaching 20,000 nmol PNPP hydrolyzed per minute per mg. This value is comparable to the highest lipid-stimulated PNPP activity reported by others for PP5 [Kang et al., 2001] indicating that the PP5 activities reported here accurately reflect the full potential for PP5 activity in cells.

Immunofluorescence

Cells plated on glass coverslips were infected with adenovirus and treated with OA as described in the legend to Figure 5A. They were fixed in 3% paraformaldehyde in PBS at room temperature for 20 min, and permeabilized with 0.1% triton X-100 in IF block buffer (1% BSA, 0.5% normal goat serum, 0.05% saponin, 25 mM Tris, 120 mM NaCl, pH 7.6). Antibodies were diluted in IF block buffer for staining. PBS was used for washing initially, and IF rinse buffer (0.1% BSA, 0.05% normal goat serum, 0.05% saponin, 25 mM Tris, 120 mM NaCl, pH 7.6) was used after permeabilization. Samples were then incubated with secondary antibodies conjugated with fluorescein or rhodamine (Molecular Probes). Following a final rinse in PBS, the coverslips were mounted using Vectashield containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA), and viewed under a Leica microscope equipped for digital photography (Hamamatsu Orca-ER camera). Image analysis was performed using Openlab (Improvision) and Photoshop (Adobe) software.

RESULTS

Low Concentrations of Okadaic Acid Stimulate a p53 Response in Proliferating T51B Cells

P53 activation anchors a robust cellular response to many types of genotoxic and replicative stressors and may result in apoptosis, G_1 arrest, or G_2 arrest [Meek, 2004; Harris and Levine, 2005; Lu, 2005]. Previous studies in T51B rat liver cells indicated a very specific dose range of the phosphatase inhibitor OA was required for optimal tumor promotion activity; similar concentrations activated p53 and blocked S-phase entry in quiescent cells treated with epidermal growth factor [Messner et al., 2001]. Suboptimal doses were without measurable effect, while higher doses were toxic. In contact-arrested T51B cells, or in other cell types that have been serum starved, OA treatment induces exit from G_0 [Afshari and Barrett, 1994; Messner et al., 2001]. It is, therefore, possible that p53 is activated in response to cell cycle entry under inappropriate conditions, rather than inhibition of phosphatases that act on the p53 pathway. As shown in Figure 1, p53 is also activated in proliferating T51B cells treated with 4–8 nM OA, as judged by the cellular content of p53, p53 phosphorylated at ser15, and p21 (cip1). Increased cyclin E and decreased cyclin A is evidence for arrest in late G_1 (Fig. 1). These points suggest OA-induced p53 activation is not obligatorily linked to events occurring during exit from G_0. Importantly, low nanomolar concentrations of OA have fewer cellular effects than do higher levels more commonly studied. For example, treatments outlined in Figure 1 did not increase levels of phosphorylated (active) p38, a MAP kinase family member known to be activated by higher levels of OA [Moriguchi et al., 1996]. The identity and extent of phosphatase inhibition resulting in the effects shown in Figure 1 are relevant to p53 regulation and to the early events of tumor promotion in the T51B cell model.

Selective Blockade of PP2A Mimics the p53-Related Responses of T51B Cells to OA

Of the phosphatases implicated in p53 regulation, PP2A and PP5, but not PP1, are sensitive

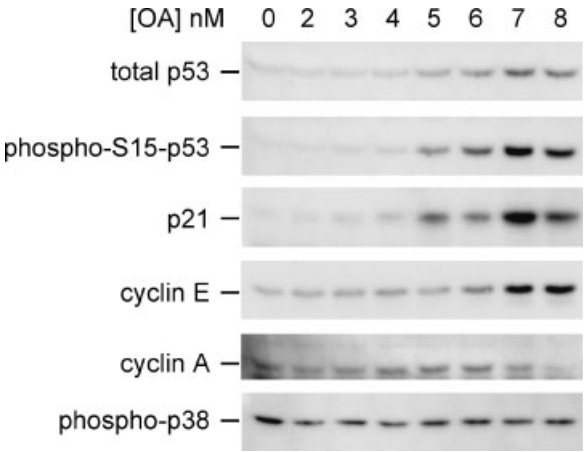

Fig. 1. Activation of the p53 pathway in T51B cells treated with low nanomolar levels of OA. Subconfluent cells treated with the indicated concentration of OA (2–8 nM) for 72 h were harvested for SDS–PAGE and analyzed by Western blot with antibodies specific for total p53, phospho-ser15-p53, p21 (cip1), cyclin E, cyclin A, and phospho-thr180-tyr182-p38. Unchanging phospho-p38 levels indicate that equal amounts of sample protein were loaded for each condition.

to blockade by the concentration range of OA examined in Figure 1. The observed titration of p53 activation by OA (increasing from 4 to 8 nM) is most consistent with the inhibition of PP5 based on in vitro data. However, OA has complex pharmacokinetic properties [Shenolikar, 1994; Namboodiripad and Jennings, 1996], making it difficult to precisely obtain a desired intracellular concentration. Although this is minimized by long incubation times (72 h in Fig. 1), distinguishing PP5 from PP2A in cells is difficult with OA alone. Therefore, as a first step in distinguishing between PP2A and PP5, we bypassed the pharmacokinetic difficulties associated with OA by using a different phosphatase inhibitor that exhibits a greater difference in potency toward these enzymes.

Fostriecin is an antitumor agent that inhibits PP2A with an IC_{50} near 5 nM [Jackson et al., 1985; Walsh et al., 1997]. Much higher concentrations are required to inhibit PP5 (IC_{50}, 70 μM) [Honkanen and Golden, 2002]. If effects of low nanomolar concentrations of OA are due primarily to inhibition of PP2A but not PP5, then fostriecin at low micromolar levels should elicit a similar response. At these concentrations fostriecin also is not expected to significantly inhibit PP1 (IC_{50} near 50 μM [Honkanen and Golden, 2002]) or DNA topoisomerase II (IC_{50} near 40 μM, [Boritzki et al., 1988]). Time course experiments indicated the onset of fostriecin's p53 effects occurred more rapidly than for OA and did not accumulate with time (data not shown). Fostriecin enters cells rapidly but is short-lived, while OA diffuses into cells and reaches equilibrium more slowly, but is more stable.

As shown in Figure 2A, treatment of T51B cells with fostriecin elevated both p53 phosphorylated at ser15 and p21 (cip1). In addition, effects of fostriecin on the levels of phosphatase protein mirror effects of OA. Both OA and fostriecin increased PP2A but not PP5 (Fig. 2). It is worth noting that most of the PP2A is likely inactivated, as large quantities of each inhibitor are present outside the cells at a diffusible concentration over 100 times higher than the estimated IC_{50}'s for PP2A. Increased PP2A protein is an indicator of drug entry into the cells. The constant PP5 protein levels in all samples indicate that changes in PP2A, p21 (cip1), and phospho-ser15-p53 are not a result of non-specific toxic effects or variation in sample loading. The magnitude of effects caused by 10 μM fostriecin was comparable to those caused by 7 nM OA (Fig. 2A). Since PP5 inhibition would require much higher concentrations of fostriecin, this result indicates that PP2A inhibition is sufficient for activation of p53 in T51B cells.

Concentrations of fostriecin, as well as OA, that inhibit PP2A also inhibit the PP2A-related phosphatase PP4 [Honkanen and Golden, 2002]. In T51B cells, levels of PP4 protein were increased by either fostriecin or OA (not shown). Although PP4 has not been implicated directly in p53 regulation, it was possible that OA and fostriecin effects were due to inhibition of this (or another related) enzyme. We therefore also tested SV40 small t-antigen, which is a selective perturbant of PP2A that displaces specific B subunits from PP2A and alters its substrate selectivity [Janssens and Goris, 2001]. Adenovirus-based expression of SV40 small t-antigen in T51B cells caused an increase in phospho-ser15-p53, as well as an increase in expression of both p21 (cip1) and a second p53-induced gene product, mdm2 (Fig. 2B). These effects overshadowed a small, but detectable effect of control adenovirus on phospho-ser15-p53 and p53 activity, which has also been observed by others [McPake et al., 1999]. Most importantly, selective disruption of PP2A with small t-antigen was sufficient to generate OA-like p53 effects in T51B cells.

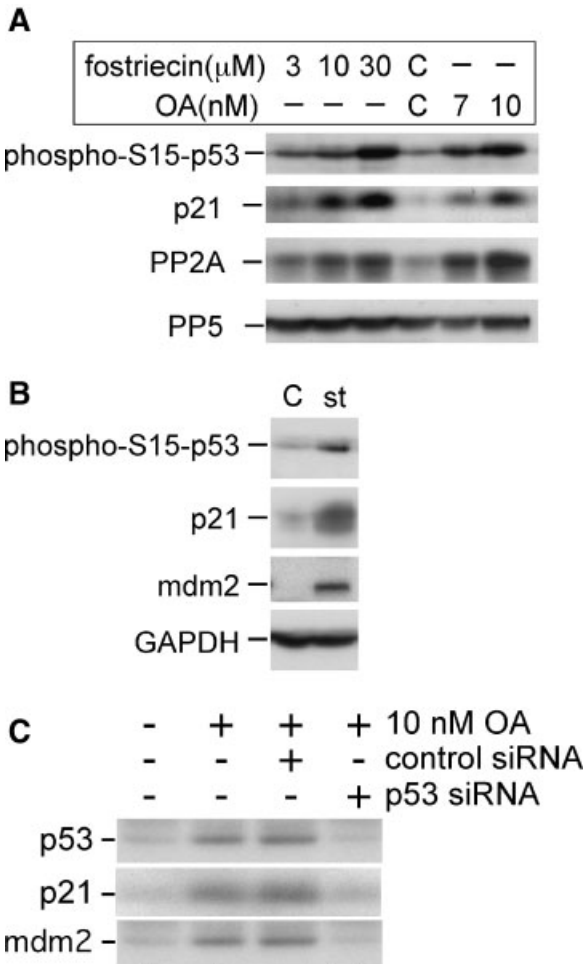

Fig. 2. PP2A-selective agents mimic OA effects on p53. **A**: Subconfluent cells were treated with vehicle (C), fostriecin (3, 10, 30 μM) for 8 h, or OA (7, 10 nM) for 48 h as indicated. Following treatment the cells were harvested and analyzed by SDS–PAGE and Western blot for phospho-ser15-p53, p21 (cip1), PP2A, and PP5. Comparable effects were observed in two or more independent experiments for each protein. **B**: Cells were infected with replication-deficient adenovirus containing the SV40 small t-antigen (st) or control adenovirus (C), each at moi = 6 for 24 h, and then fresh media was added for an additional 42 h. Cells were then harvested and analyzed by SDS–PAGE and Western blot for phospho-ser15-p53, p21 (cip1), and mdm2. GAPDH was probed as a control for equal gel loading of samples. Similar results were obtained in three independent experiments. **C**: Control cells or cells transfected with either 20 nM siRNA specific for p53 (p53 siRNA) or a negative control sequence (control siRNA) were grown for 2 days, then treated with or without 10 nM OA for an additional 2 days. Cell lysates were then prepared and analyzed by SDS–PAGE and Western blot for p53, p21 (cip1), and mdm2. Similar results were obtained in two independent experiments.

Levels of p21 (cip1) can be regulated by multiple mechanisms besides p53 [Gartel and Tyner, 1999]. To be used as an effective reporter of OA-induced p53 activity in T51B cells, it was important to establish that the observed changes in this protein (as well as in mdm2) are dependent on the presence of p53. They should not occur following p53 knockdown. Decreased expression of specific proteins in mammalian cells can be achieved using small interfering RNAs (siRNA, [Elbashir et al., 2001]). As shown in Figure 2C, increases in both p21 (cip1) and mdm2 by OA were specifically inhibited by pre-treatment of cells with siRNA specific for p53. This verifies that increased levels of these two proteins reflect an increase in p53 activity in this study.

Knockdown of PP2A, but not PP5, Is Sufficient to Increase p21 (cip1) in T51B Cells

Figure 3 shows that treatment of T51B cells for 3 days with siRNA specific for the catalytic subunit of PP2A resulted in decreased PP2A protein and elevated p21 (cip1) levels. This occurred without significant change in PP4 or PP5, indicating the siRNA treatment was specific for PP2A. We did not observe a comparable elevation of phospho-ser15-p53 with PP2A knockdown (data not shown). This may be due to

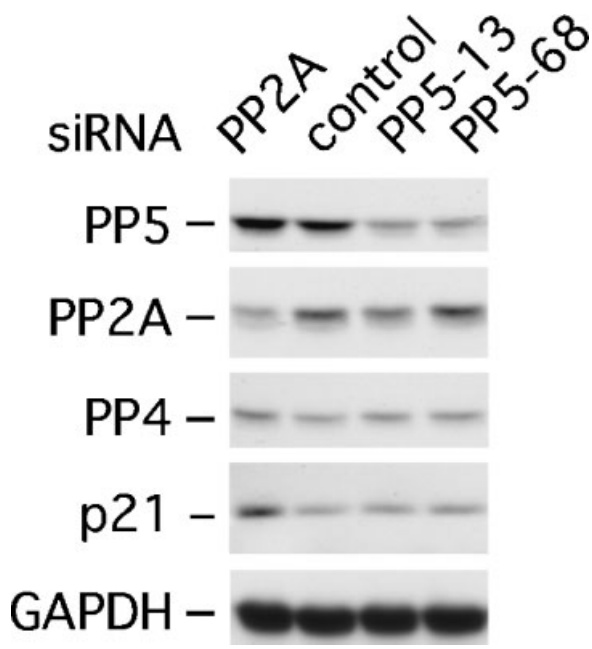

Fig. 3. Effect of siRNA-mediated knockdown of PP2A or PP5 on p21 (cip1) levels in T51B cells. Cells subjected to mock transfection (control) or transfection with 10 nM of the indicated siRNA were harvested 3 days later and lysates analyzed by SDS–PAGE and Western blot for PP5, PP2A, PP4, and p21 (cip1). GAPDH was also probed as a loading control. Induction of p21 (cip1) proportional to the decrease in PP2A levels, but not PP5 levels, was seen in two or more independent experiments.

the fact that the knockdown causes a less severe loss of PP2A activity than does OA treatment, or it may reflect the fact that changes in p21 (cip1) levels are sustained much longer than changes in p53 protein [Chen et al., 1998]. For this reason, p53-induced gene products may be more robust indicators of p53 activation than are p53 phosphorylation state or p53 protein levels. The increased p21 (cip1) seen in Figure 3 is consistent with activation of p53 caused by siRNA-induced loss of PP2A.

In contrast, when two distinct siRNAs were used to decrease PP5 expression, there was essentially no effect on p21 (cip1) levels in T51B cells (Fig. 3). PP2A and PP4 levels were unchanged by these treatments, indicating the siRNAs were specific. Quantification showed that a 50–70% reduction of either PP5 or PP2A expression was typically achieved by siRNA treatment in these experiments. Although we cannot exclude the possibility that complete loss of PP5 would trigger p53 activation, the p53 pathway appears more sensitive to loss of PP2A than PP5.

Activation of p53 by Okadaic Acid Occurs in Cells Expressing OA-Resistant PP5

The best approach to eliminating PP5 as a mediator of OA-induced p53 activation is to examine the OA response of T51B cells under conditions in which PP5 remains active. To accomplish this we designed an OA-resistant form of PP5. The structural basis of OA binding and inhibition has been examined for PP1 [Zhang et al., 1996]. In that study, Y272 of PP1 was particularly important for OA sensitivity. This amino acid likely forms a hydrogen bond with the OA carboxylic acid group known to be critical for phosphatase inhibition [Maynes et al., 2001]. For PP5, the corresponding residue is Y451. When flag epitope-tagged proteins were expressed and purified from bacteria, PP5-Y451A had similar specific activity as wild-type PP5, but the apparent IC_{50} for OA increased approximately 500-fold from the 10 nM seen for the wild-type enzyme (Fig. 4A). In a separate study in which PP5 was shown to mediate hormonal activation of a potassium channel, heterologous expression of flag-PP5-Y451A rescued this response from blockade by OA [Gentile et al., 2006], demonstrating that this construct can functionally replace endogenous PP5 in cells. Analysis of flag-PP5-Y451A following transient over-expression in T51B cells is shown in Figure 4B. The activity of immunoprecipitated PP5 in the presence of 500 nM OA was $72 \pm 6\%$ (mean $\pm$ SE) of the activity in the absence of OA. In contrast, when flag-tagged

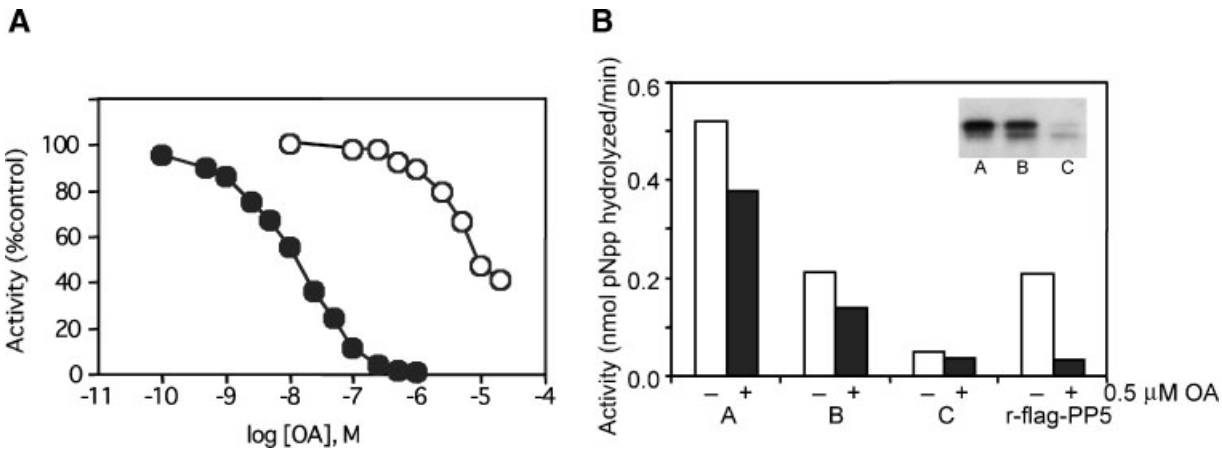

Fig. 4. Characterization of PP5-Y451A. **A**: Inhibition of recombinant flag-PP5 and flag-PP5-Y451A by OA. PP5 variants expressed as GST fusion proteins were purified from *E. coli* and assayed for phosphatase activity in the presence of increasing OA concentration. Activity is expressed as percent of control (no OA added) for flag-PP5-WT (solid circles) or flag-PP5-Y451A (open circles). The assay was performed in triplicate; the same results were obtained in three independent assays performed under identical conditions. **B**: Expression of flag-PP5-Y451A in T51B cells. Cells were transfected with pCI-flag-PP5-Y451A: (A) (3 μg DNA); (B) (1.5 μg DNA); (C) (0.75 μg DNA). After 2 days cell lysates were prepared and aliquots of PP5 immunoprecipitates were analyzed by phosphatase assay and by Western blot for PP5 (inset). The signals (–/+ OA) from comparable aliquots of untransfected cells (activity below 0.01 nmol/min) were subtracted from each point. For comparison, 20 ng recombinant wild-type flag-PP5 expressed and purified from bacteria was immunoprecipitated and analyzed in parallel (rflag-PP5). Activity was measured in the absence (open bars) or presence (black bars) of 0.5 μM OA, and results are expressed as nmol PNPP hydrolyzed per minute. OA-resistant PP5 activity was documented twice in T51B cells.

recombinant wild-type PP5 was subjected to immunoprecipitation and assayed in parallel, activity was decreased to 11% by 500 nM OA. We conclude that expression of flag-PP5-Y451A by chemical transfection yields active PP5 that is resistant to inhibition by OA.

Prevention of PP5-mediated and OA-induced p53 activation in a population of cells requires that a high fraction of the cells express flag-PP5-Y451A. Chemical transfection of T51B cells is not sufficiently efficient to achieve this, so an adenoviral-mediated delivery system was developed. This technique also has the advantage of cell cycle-independent delivery and protein expression not seen with chemical transfection methods. Characterization of PP5-Y451A expression in T51B cells infected with AdV-flag-PP5-Y451A is shown in Figure 5. While infection at a low moi (0.4 pfu/cell) gave a small fraction of the cells over-expressing PP5 levels (Fig. 5A, panel B), a higher concentration of adenovirus (moi = 4) resulted in elevated PP5 in nearly every cell (Fig. 5A, panel A). After 24 h, PP5 activity in the infected cell population was at least 600-fold higher than endogenous PP5, and it remained elevated for several days following infection (Fig. 5B). This high level argues that even though expression is heterogeneous (Fig. 5A), most individual cells contain more OA-resistant PP5 than endogenous OA-sensitive PP5. Consequently, PP5 is removed as a cellular target of OA; the relationship between block of PP2A and the p53-related effects of 10 nM OA can now be investigated more specifically.

Figure 6 shows the effect of 10 nM OA on cells expressing flag-PP5-Y451A, and on cells infected with a control replication-deficient adenovirus. In both cases, OA increased levels of total p53 and of p53 phosphorylated at ser15. OA also caused increases in two p53 responsive gene products, p21 (cip1) and mdm2. Although, as observed in Figure 2B, there was a small effect of control adenovirus itself, the magnitude of the OA-induced increase beyond the adenoviral control was the same as that seen in uninfected cells. Flag-PP5-Y451A expression from the adenoviral vector was not altered by 10 nM OA. Quantification of the changes in total p53 and in p53 phosphorylated at ser15 from four replicate experiments using an Odyssey infrared imaging system showed that OA effects were the same (within 20%) in cells expressing flag-PP5-Y451A as in uninfected or virus only control cells. Similar quantitative analyses of change in p21 (cip1) levels from

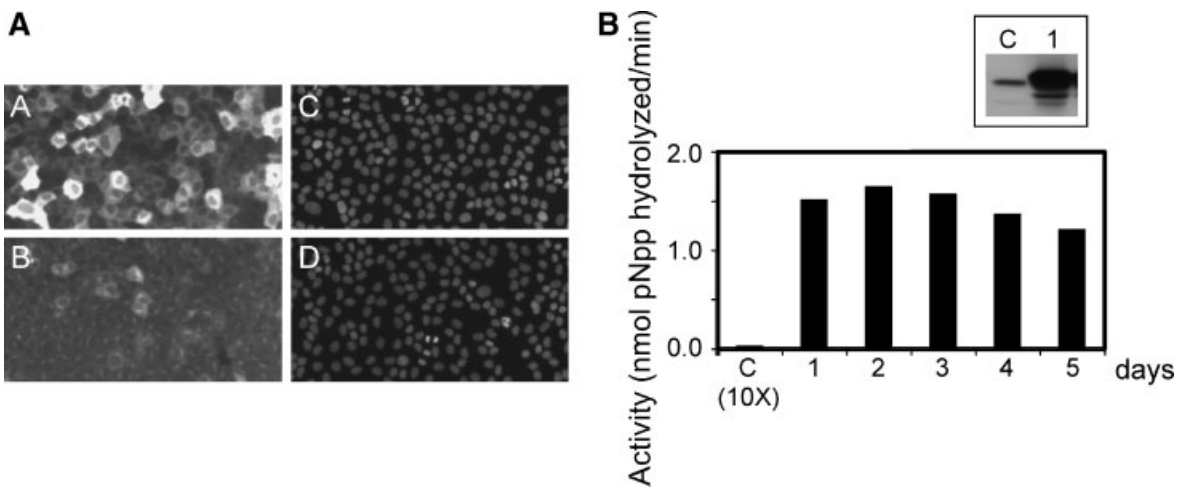

Fig. 5. Adenoviral-mediated expression of flag-PP5-Y451A in T51B cells. **A**: Analysis of AdV-flag-PP5-Y451A-infected cells by indirect immunofluorescence. Cells treated with adenovirus at moi 4 pfu/cell (**A** and **C**) or moi 0.4 pfu/cell (**B** and **D**) were fixed 2 days later and processed for immunolocalization of PP5 (A and B) as described under Materials and Methods. The nuclear stain DAPI was used to identify individual cells (C and D). Results shown correspond to optimal exposure times for the PP5 fields of 0.2 s (at moi = 4) and 0.6 s (at moi = 0.4). **B**: Time course of PP5 expression. T51B cells were infected with AdV-flag-PP5-Y451A (20 μl crude AdV lysate per 100-mm dish; estimated moi 2 pfu/cell) and analyzed for PP5 activity after infection for 1–5 days. A sample from uninfected cells (C, 10×) corresponds to 10-fold more starting protein compared to infected cells. The inset shows a PP5 Western blot of 10 μg extract from uninfected cells (C) or cells infected for 1 day (1). Stable, high expression of PP5 protein and activity was achieved 1 day post-infection in similar experiments.

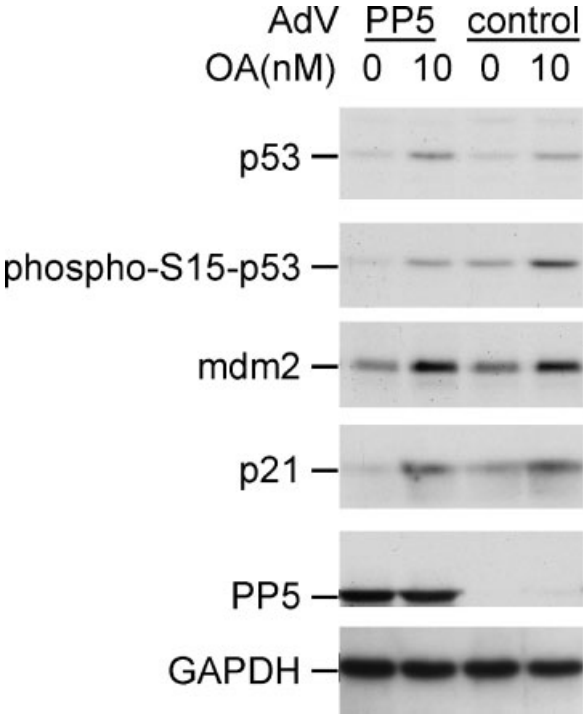

Fig. 6. Analyses of OA effects in AdV-flag-PP5-Y451A-infected T51B cells. Cells were infected with AdV vector encoding flag-PP5-Y451A (PP5) or empty AdV control (control) at estimated moi = 6.48 h post-infection, cells were treated with or without 10 nM OA for 8 h, then cell lysates prepared and analyzed by Western blot using antibodies specific for p53 (total and phospho-ser15), mdm2, p21, and PP5. GAPDH was probed as a loading control. Comparable effects on p53 levels and activity (ser15 phosphorylation, and induction of p21 and mdm2) were observed in four independent experiments.

two experiments showed cells expressing flag-PP5-Y451A or control adenovirus had the same OA-induced p53 activity (within 20%) as uninfected cells.

The biochemical observations of Figure 6 were extended by measuring OA-induced p21 (cip1) levels in individual cells over-expressing PP5-Y451A by immunofluorescence. Most importantly, OA-induced p21 (cip1) was observed in cells having the highest PP5-Y451A (Fig. 7). Thus, higher PP5-Y451A expression in individual cells did not result in lower p21 (cip1) induction by OA in those cells, contrary to what would be expected if PP5 inhibition were required. Although a slight increase in p21 (cip1) was observed in control cells expressing the highest PP5-Y451A levels, the effect of OA was significantly greater. Effects of 10 nM OA in T51B cells expressing OA-resistant PP5 establish that p53 is activated independently of PP5 inhibition.

DISCUSSION

The high sensitivity of PP2A to OA and fostriecin, together with the effects of small t-antigen expression and PP2A knockdown, implicate PP2A as the target for OA-induced p53 activation and late G_1 arrest in T51B cells. In contrast, reduction of PP5 using siRNA failed to mimic the effects of OA on p53-related events, and over-expression of an OA-resistant form of

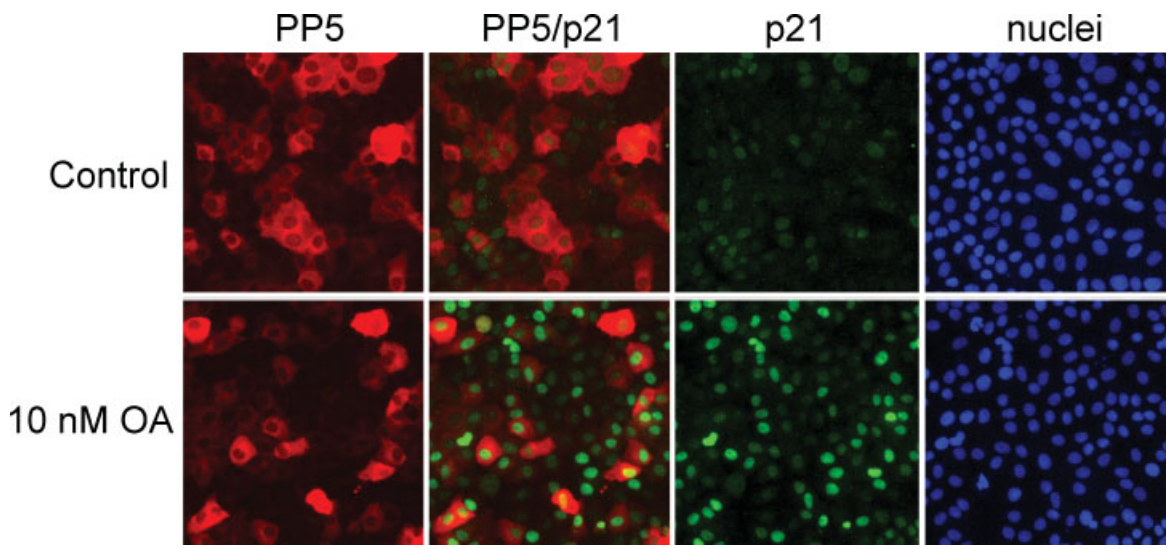

Fig. 7. Effect of 10 nM OA on cellular content of p21 (cip1) in AdV-flag-PP5-Y451A-infected T51B cells. Cells infected with AdV vector encoding flag-PP5-Y451A (estimated moi = 6) were cultured for 48 h with or without 10 nM OA. Representative fields of roughly 80 cells are shown for each of these two conditions after triple staining with the nuclear dye DAPI (blue), PP5 antibodies (red), and p21 (cip1) antibodies (green) as described in Materials and Methods.

PP5 did not prevent OA-induced activation of p53.

Increases in p53 protein, p53 phosphorylation state, and p53 activity were observed at specific non-toxic concentrations of OA (4–8 nM) compatible with long-term culture of T51B cells [Messner et al., 2001]. These effects resulted from selective inhibition of PP2A or PP2A-like phosphatases, despite increases in phosphatase protein. Support for this central point comes from considering the unique properties of OA and PP2A in intact cell experiments. Studies on the membrane permeability of OA revealed that long times are needed for equilibration of the cytoplasm with the extracellular medium lNamboodiripad and Jennings, 1996]. Relevant factors include poor membrane permeability of OA at pH 7.2, and the high intracellular level of PP2A compared to the low concentration of OA required for its inhibition: cells contain micromolar levels of PP2A [Cohen et al., 1990] that, in vitro, bind OA with an IC_{50} in the range of 0.1 nM OA [Honkanen and Golden, 2002]. Continued influx of OA over the 48 h time point used in these experiments increasingly drives PP2A to the OA-bound state. Physiological effects require influx of sufficient OA to block a "significant" quantity of PP2A. Thus, a fixed amount of OA influx must occur, and the time required is inversely proportional to the extracellular concentration. At 10 nM OA, this leads to a slow onset of PP2A inhibition that is accompanied by increased levels of PP2A protein, due to increased synthesis and/or decreased degradation. However, OA is a long-lived inhibitor, and so total PP2A activity in cells almost certainly decreases during OA treatment.

PP2A must not be completely inhibited, as it is an essential enzyme whose activity influences numerous cellular processes. Complete knockout of the PP2A catalytic subunit is lethal [Gotz et al., 1998]. In our experiments, increased PP2A levels may allow the cells to survive by protecting against doses of OA that would otherwise completely block the phosphatase. Although some essential level of total activity is maintained, there must be a significant decrease in the specific activity of PP2A (measured as activity/amount PP2A catalytic subunit protein). Such a decrease in active catalytic subunit would dilute its productive interactions with substrates and/or targeting subunits; this could account for the p53 effects we observe. Further analyses of changes in phosphatase biochemistry resulting from this non-lethal protocol may also reveal why prolonged exposure of T51B cells to OA results paradoxically in tumor promotion rather than tumor suppression [Messner et al., 2001].

Our experiments implicate PP2A as a significant negative regulator of basal p53 phosphorylation state and p53 activity. We cannot rule out a similar role for the PP2A-related phosphatases, PP4 and PP6. PP4 has sensitivity profiles for OA and fostriecin that are similar to PP2A [Honkanen and Golden, 2002], and so its inhibition may contribute to p53 effects induced by those pharmacological reagents. However, at present there are no data that specifically implicate PP4 or PP6 in p53 activation, in contrast to the PP2A-specific effects of small t-antigen and PP2A siRNA. As PP2A inhibition is sufficient for p53 activation in T51B cells, the simplest interpretation is that PP2A inhibition mediates p53 activation by OA.

Although our data argue against a role for PP5 in p53 regulation in non-neoplastic T51B cells, this differs from what others postulated from studies done in tumor cells [Zuo et al., 1998, 1999; Urban et al., 2003]. Studies implicating PP5 as a negative regulator of p53 function were performed in A549 lung carcinoma cells and derivatives of a p53-deficient fibroblast line [Zuo et al., 1998]. Several points make it unclear whether p53-related events in these cells are relevant to those occurring in phenotypically normal cells. A549 cells lack ARF, a key inhibitor of mdm2, and consequently have a dampened p53 response [Ries et al., 2000; Lu, 2005]. This limits the ability of the cells to quench oncogene-induced cell cycle progression and likely impacts other facets of p53 regulation [Alarcon-Vargas and Ronai, 2002]. Over-expression of p53 in engineered cells is not expected to fully recapitulate all of the regulatory intricacies present in normal, wild-type cells. More generally, others have shown that the status of post-translational modifications for wild-type p53 can be altered by cell transformation [Bode and Dong, 2004]. Additionally, p53 activation in A549 cells appears linked to glucocorticoid receptor activation resulting from PP5 knockdown [Zuo et al., 1999; Urban et al., 2003]. Glucocorticoid receptor action and p53 activation can be cell and tissue type specific [Gottlicher et al., 1996; Jenkins et al., 2001; Lu, 2005]. Dexamethasone,

a glucocorticoid agonist, does not activate p53 in T51B cells (DM and SR, unpublished). For these reasons it is likely that differences in cell type contribute to the differing results observed in the present study compared to previous reports, with respect to the role of PP5 in maintaining the basal phosphorylation of p53. The situation appears more complex, however, as more recent studies by a third group found no effect of PP5 knockdown on basal p53 levels or p53 phosphorylation at ser15 in A549 cells [Ali et al., 2004]. These investigators assigned a signal transduction role to PP5, showing that removal of PP5 impaired DNA damage response signaling to p53. Clearly, a full understanding of the relationship between PP5 and p53 remains to be determined. Nevertheless, in resting T51B cells, a phenotypically normal tumor promotion model system, manipulations decreasing PP2A activity correlated best with p53 responses caused by low concentrations of OA, whereas manipulation of PP5 activity or expression had no effect.

The effect of PP2A on p53 activation may be direct, as PP2A has been shown to dephosphorylate p53 in vitro [Scheidtmann et al., 1991], or indirect, as PP2A is known to control numerous pathways that can alter the stability and activation of p53 [Schonthal, 2001; Fernandez et al., 2002; Bode and Dong, 2004]. In one of the better characterized examples of the latter point, phosphorylation of the p53 regulator mdm2 is regulated by PP2A [Alarcon-Vargas and Ronai, 2002]. Association of mdm2 with cyclin G and PP2A is thought to be required for mdm2 activation [Okamoto et al., 2002]. Inhibition of PP2A in this context is predicted to decrease p53 degradation triggered by mdm2 ubiquitin ligase activity. Elevated p53 levels and activity, as we observed, would result. Similar effects are expected from increased p53 kinase activity at ser15. Phosphorylation at this site is the first step in a series of events to increase the half-life of p53 protein [Meek, 2004]. It facilitates phosphorylation of adjacent sites that block interactions with mdm2, and stabilize p53 association with the co-activator p300. There are several kinases known to be active at ser15, including those involved in the DNA damage response function of p53. It will be important to determine whether inhibition of PP2A by OA activates one or more of these kinases. In addition, the phosphorylation status of other p53 sites thought to regulate DNA binding or influence the selectivity of p53 responsive gene transcription is unknown. This is a complex area that will require additional study. Disruptions in post-translational regulation of the p53 protein are a central theme in each of these possible mechanisms. We propose that activation of p53 in OA-treated cells results from disrupted PP2A-dependent regulation at one or more points that may include upstream p53 kinases, the p53 protein itself, or downstream regulators of p53 half-life.

In addition to G_1 arrest, OA-triggered cell cycle entry [Messner et al., 2001] may also be mediated by inhibition of PP2A. Small t-antigen induces cell cycle entry accompanied by MAP kinase activation and increased AP-1 activity [Howe et al., 1998]. More recently, PP2A was reported to bind p130, the Rb family protein that regulates cell cycle progression from G_0 to G_1 in many cell types [Vuocolo et al., 2003]. Phosphorylation of p130 by cdk2 and cdk4 releases bound E2F proteins and triggers entry into G_1. PP2A was shown to dephosphorylate critical sites within p130, contributing to increased p130 levels and growth inhibition by all trans-retinoic acid [Vuocolo et al., 2003]. The prediction from these observations, that OA treatment reduces p130 levels and causes cell cycle entry, was reported by us in T51B cells previously [Messner et al., 2001]. These reports, together with our data that exclude PP5 and implicate PP2A in the p53 effects of OA, support a model for progression of quiescent cells to late G_1 arrest based primarily on inhibition of PP2A.

PP2A actually constitutes a large and complex subfamily of phosphatases containing a catalytic subunit combined with scaffolding A subunit and one of many B subunit forms, which control both localization and substrate activity. Additional regulatory proteins can combine with specific forms of trimeric PP2A or supplant certain B subunit forms [Janssens et al., 2005]. Recent studies have begun to make progress delineating the role of specific PP2A forms in defined signal transduction pathways [Chen et al., 2004; Strack et al., 2004]. For example, although small t-antigen blocks many PP2A forms, Chen and colleagues found that PP2A containing B56γ was the sole critical target of small t-antigen during transformation. Whether functional dilution of active B56γ-containing complexes accounts for tumor promotion by OA is unknown. Similarly, it is unknown whether a single, specific form of

PP2A is responsible for OA-induced p53 activation and late G_1 arrest in cells. Identification of PP2A as the primary target phosphatase in OA-induced p53 activation establishes the framework for future experiments to address these issues with respect specifically to tumor promotion.

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