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Ser/Thr Protein Phosphatase Type 5 (PP5) Is a Negative Regulator of Glucocorticoid Receptor-Mediated Growth Arrest†

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ABSTRACT: Ligand-induced glucocorticoid receptor (GR) activation has recently been linked to the inhibition of cell proliferation via the transcriptional induction of $p21^{WAF1/Cip1}$, which functions as a universal inhibitor of cyclin-dependent protein kinases. Herein, we identify a Ser/Thr protein phosphatase (PP5) that promotes cellular proliferation by inhibiting both glucocorticoid and p53-mediated signaling pathways leading to $p21^{WAF1/Cip1}$-mediated growth arrest. The suppression of PP5 expression (1) markedly increases the association of GR with its cognate DNA-binding sequence, (2) induces GR transcriptional activity without the addition of hormone, and (3) increases dexamethasone-mediated induction of GR reporter activity to a level that is ~10 times greater than the maximal response obtainable in the presence of PP5. PP5 has no apparent effect on the binding of hormone to the GR, and dexamethasone-mediated growth arrest correlates with an increase in p53 phosphorylation. Comparative studies in p53-wild-type, p53-defective, and p53-deficient cell lines indicate that either (1) p53 participates in GR-mediated induction of $p21^{WAF1/Cip1}$, with the hyperphosphorylation of basal p53 induced by glucocorticoids sufficient for the propagation of an antiproliferative response when PP5 expression is inhibited, or (2) PP5 acts where p53-mediated and GR-induced signaling networks converge to regulate the transcriptional induction of $p21^{WAF1/Cip1}$. Thus, aberrant PP5 expression may have an additive effect on the development of human cancers by promoting cell proliferation via the inhibition of a GR-induced antiproliferative signaling cascade, and facilitating neoplastic transformation via the inhibition of a growth-arresting p53-mediated response that guards against genomic instability.

Glucocorticoids are essential hormones that influence a wide spectrum of cellular functions (*1*). In many types of cells, glucocorticoids produce an antiproliferative response that is associated with the accumulation of cells prior to the onset of DNA replication. The glucocorticoid response is initiated in the cytoplasm by the interaction of hormone with a cognate intracellular receptor complex. Upon glucocorticoid

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binding, the activated GR enters the nucleus, where it acts as a ligand-activated transcriptional stimulator or repressor of primary response genes by binding to glucocorticoid hormone-responsive elements (GRE) in the promoter regions of steroid-responsive genes. Hormone-activated GR can also affect transcription by direct protein−protein interactions with other transcription factors and accessory proteins (for review, see refs *1*−*5*). The ability of glucocorticoids to induce growth suppression appears to arise from complex signaling networks in which glucocorticoid receptor-mediated transcription of primary response genes regulates the subsequent expression or activity of a diverse set of downstream proteins, some of which regulate cell cycle progression (*3*, *4*). In addition, "cross talk" between hormone-regulated signaling networks and signal transduction cascades induced by extracellular regulators, such as growth or differentiation factors, may complement or compete with steroid-dependent processes (*2*−*6*).

The molecular mechanism(s) by which GR activation leads to growth arrest is (are) unclear. Several studies, however, suggest that GR-induced signaling networks that impede cellular proliferation target processes that regulate progression through the G1/S-phase transition (*7*−*9*). One apparent target of GR-induced signaling networks is a family of cyclin-dependent protein kinases (CDKs).[1] CDKs are key regulators of cell cycle progression, controlling both signaling cascades which stimulate normal cell cycle progression and checkpoint control mechanisms that inhibit DNA replication (*10*−*12*). Normal progression through the G1/S phase of the cell cycle requires a temporal increase in G1-CDK activity. Increased CDK activity is elicited by both growth factors (i.e., EGF) and growth-inducing steroid hormones (i.e., estrogen), with CDK activity regulated ultimately by the association with cognate cyclins (D1, D2, D3, and E) and by reversible phosphorylation (*9*−*12*). CDK activity is also regulated in a negative manner by the association with CDK inhibitor proteins, such as $p21^{WAF1/Cip1}$, which target the CDKs, cyclins, or CDK/cyclin complexes (*13*, *14*). Transcription factors associated with a variety of growth inhibitory signaling cascades [i.e., the p53 tumor suppressor protein (*13*−*18*), the retinoic acid receptor (*19*), AP2 (*20*), E2A (*21*), and C/EBPα (*22*)] regulate $p21^{WAF1/Cip1}$ transcription through their interaction with respective response elements in the $p21^{WAF1/Cip1}$ promoter. The $p21^{WAF1/Cip1}$ protein then induces G1 growth arrest and blocks entry into S-phase by inactivating G1-CDKs (*13*, *14*) and/or by inhibiting the activity of proliferating cell nuclear antigen (*23*). Recent studies in fibroblasts (*7*), osteoscarcoma (*8*), and rat hepatoma cells (*9*) have shown that dexamethasone induces the expression of $p21^{WAF1/Cip1}$. This suggests that $p21^{WAF1/Cip1}$ may be a critical participant of the GR-induced signaling network(s) that regulate(s) G1-growth suppression.

Since phosphorylation is associated with virtually all known examples of cell cycle regulated transcriptional control, it seems likely that protein phosphatases participate in the regulation of signaling networks that control cell cycle progression. In mammals, at least 13 closely related serine/threonine protein phosphatases (PPases) have been identified (for review, see refs *24* and *25*), and several (i.e., PP1, PP2A, PP4, and PP5) are sensitive to natural toxins, such as okadaic acid (*26*), microcystin (*27*), and fostriecin (*28*, *29*). Reports indicating that okadaic acid has tumor promoting activity (*31*, *32*) and that fostriecin has antitumor activity (for review, see ref *32*) have led to speculation that the toxin-sensitive PPases regulate cell growth (*24*, *26*, *29*−*31*). However, because there are no truly type-specific inhibitors of the known PPases and the physiological substrates for PP4, PP5, and PP6 are unknown, studying the roles of individual PPase in the control of cell cycle progression has proven difficult.

To more precisely define the roles of individual PPases in the control of cell cycle progression, we are in the process of developing antisense oligonucleotides (ODNs) targeting each of the known human PPases. Studies with ISIS 15534, a second-generation chimeric antisense oligonucleotide that specifically inhibits the expression of human PP5 (*33*), revealed that the inhibition of PP5 expression in A549 cells results in G1-growth arrest with a corresponding increase in the expression of $p21^{WAF1/Cip1}$ (*33*). Previous studies have shown that PP5 coimmunoprecipitates with the GR, suggesting that PP5 is a component of the GR−heat shock protein 90 (hsp90) complex (*34*, *35*). Therefore, when an association between dexamethasone-induced G1-growth arrest and an increase in the expression of $p21^{WAF1/Cip1}$ was reported (*7*), we investigated the possibility that PP5 participates in the regulation of GR-mediated growth arrest and/or "cross-talk" between stress-induced, p53-mediated signal transduction cascades and GR-mediated transcriptional activity. In this study, we demonstrate that, in addition to its effects on p53-mediated induction of $p21^{WAF1/Cip1}$, PP5 also acts as an inhibitor of GR-mediated signaling networks leading to the induction of $p21^{WAF1/Cip1}$ and growth arrest. Together the findings presented suggest that PP5 is a key regulator of antiproliferative signaling cascades controlling cell cycle progression in A549 lung carcinoma cells.

EXPERIMENTAL PROCEDURES

Cell Lines. All cell lines were human and routinely passed when 90−95% confluent unless indicated otherwise. T-24 bladder carcinoma cells and A549 lung carcinoma cells were obtained from the American Type Tissue Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. TR9-7 cells were kindly provided by Drs. G. Stark and M. Agarwal (Cleveland Clinic Foundation). TR9-7 cells were derived from the $p53^{-/-}$ human fibroblasts (MDAH041) and contain tetracycline-regulated transactivator and operator plasmids to control the expression of wild-type p53 (*36*). TR9-7 cells were maintained in DMEM containing 10% FBS, 600 μg/mL G-418, 1 μg/mL hygromycin, and 2 μg/mL tetracycline (to inhibit the expression of p53). TR9 cells were derived from TR9-7 cells via reverse selection in culture media lacking hygromycin, G-418, and then tetracycline. As a result, TR9 cells have lost the ability to express p53 in response to tetracycline deprivation.

Oligonucleotide Synthesis and Assay for Oligonucleotide Inhibition of PP5 Expression. 2′-*O*-(2-Methoxy)ethylphosphothioate oligonucleotides were synthesized and purified as described previously (*33*, *37*). Oligonucleotides were added to cells with Lipofectin (Life Technologies; Gaith-

[1] Abbreviations: PPase, serine/threonine protein phosphatase; PP5, Ser/Thr protein phosphatase type 5; CDK, cyclin-dependent protein kinase.

ersburg, MD), as described previously (*38*, *39*). Assessment of ISIS 15534 mediated inhibition of PP5 expression was achieved by measuring the decrease in PP5 mRNA levels with Northern analysis and protein levels with Western analysis, as described previously (*33*). Hybridization was visualized by autoradiography, and the membranes were then stripped and reprobed with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe to confirm equal loading. Quantification of hybridization signals was achieved by analysis of the scanned autoradiograms using the NIH Image program (ImagePC).

Immunoblotting of p53. Western analysis was performed essentially as described previously using anti-p53 mouse monoclonal antibody (DO-1; Santa Cruz Biotechnology Inc.; Santa Cruz, CA) generated against p53 (*33*). Briefly, A549 cells grown in T-75 flasks were washed twice with ice-cold PBS. Then, 250 μL of lysis buffer (50 mM Tris-HCl, pH 8.0, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM sodium fluoride, 1 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) was added to each flask. The extract was then subjected to centrifugation at 13000*g* for 5 min, and an aliquot of the supernatant was removed for protein determination. The remaining supernatant was added to an equal volume of 2× sample buffer (120 mM Tris-HCl, pH 7.4, 200 mM dithiothreitol, 20% glycerol, 4% SDS, and 0.02% bromphenol blue). Protein was determined using a Bio-Rad Bradford assay (Bio-Rad) with bovine serum albumin as standard. Typically 25−50 μg of protein was then separated by electrophoresis on 10% SDS−polyacrylamide gels. The gel was electrophoretically transferred to Immobilon-P membranes (Millipore) and blocked for 1 h with Tris-HCl, pH 7.4, containing 150 mM NaCl and 5% nonfat milk. To detect p53, membranes were incubated with anti-p53 antibody diluted in Tris-HCl (pH 7.6), 150 mM NaCl, 0.2% Tween 20 (TBST) containing 2% nonfat milk for 18 h at 4 °C. The membrane was then washed, and the primary antibody was detected employing ECL Western blotting detection reagents (Amersham Life Science, Buckinghamshire, England), following the protocols of the manufacturer.

Analysis of Cell Growth. A549 cells were seeded in 12-well tissue culture plates at a density of 5.0×10^4 cells/dish. The next day, the cells were treated with PP5-specific antisense oligonucleotides (ISIS 15534) or the scrambled mismatch control (ISIS 15521) at a final concentration of 300 nM as described above. At the time points indicated, the cell cultures were treated briefly with trypsin to detach the cells from the dish (three wells from each test group). The number of cells was then determined using a cell counter (Coulter Counter ZM). Cell viability was determined with trypan blue staining. The percentage of viable cells was calculated by dividing the number of cells excluding trypan blue by the total number of cells, and the results are reported as the mean ± SD of data collected from three independent experiments.

Gel Mobility Shift Assay. Nuclear extracts and gel mobility shift assays were conducted as originally described by Li et al. (*40*), adopting slight modifications described previously (*33*). Briefly, A549 cells were cultured in 60 mm dishes and treated with ISIS 15521 or ISIS 15534 antisense oligonucleotides as described above. Sixteen hours after treatment, the cells were treated with dexamethasone. Six hours later, the cells were collected, washed, and lysed as described previously (*33*). Nuclei were precipitated by centrifugation, resuspended in ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF; 100 μL per 60 μL of packed nuclei), incubated at 4 °C for 60 min with gentle shaking, and then subjected to centrifugation at 4 °C for 5 min. The supernatant was collected, aliquoted, and stored at −80 °C. Protein was determined as described above.

DNA binding reactions were performed by incubating 10 μg of each nuclear extract with 1 ng of ^{32}P-end-labeled GRE-probe (GR consensus binding sequence 5′-AGAGGATCT-GTACAGGATGTTCTAGAT-3′; Santa Cruz) and 1 μg of poly(dI-dC) in binding buffer [20 mM HEPES, pH 7.9, 0.25 M EDTA, 50 mM KCl, 1 mM DTT, 1 mM PMSF, and 10% (v/v) glycerol] at room temperature for 20 min. Anti-GR monoclonal antibody (1 μg; Santa Cruz) was then added to each reaction and incubated at room temperature for 30 min. Samples were separated by electrophoresis on a 5% polyacrylamide gel containing Tris borate/EDTA buffer and visualized by autoradiography.

Immunoprecipitation of p53 Protein. A549 cells were cultured in 60 mm dishes until the cell cultures were ∼80% confluent, washed with PBS, and placed for 1 h in phosphate-free DMEM containing 5% fetal bovine serum. The cell cultures were then treated with 100 nM dexamethasone or 300 nM ISIS 15521 or ISIS 15534 as described previously (*33*), three dishes per group, and [^{32}P]phosphate (0.2 mCi/mL) was added to the media. Six hours after dexamethasone treatment, the cells were collected, washed with ice-cold PBS, and stored at −80 °C prior to analysis. Cell lysates were prepared by incubating the thawed cells at 4 °C for 1 h in a buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM sodium chloride, 5 mM EDTA, 1 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 50 mM Tris-HCl, pH 8.0. Insoluble debris was removed by centrifugation at 3000 rpm at 4 °C for 15 min. The supernatants were collected and precleared by incubation with 1.2 μg of normal mouse IgG (Santa Cruz) and 20 μL of protein A−agarose for 1 h at 4 °C, followed by centrifugation at 1500 rpm at 4 °C for 5 min. Equal amounts of protein from each precleared lysate were incubated in the presence of 1 μg of anti-p53 mouse monoclonal antibody DO-1 (Santa Cruz) for 2 h at 4 °C. Twenty microliters of protein A−agarose was added to the mixture, which was then incubated for 16 h at 4 °C with rocking. The agarose beads were collected by centrifugation and washed 5 times with 1 mL of ice-cold cell lysis buffer. After the final wash, the pellet was resuspended in 40 μL of 2× sample buffer, boiled for 3 min, and separated by SDS−PAGE on 10% gels. The bands were visualized by autoradiography.

Receptor Binding Studies, Transfections, and Luciferase Assays. Intact cell binding studies were conducted with [^{3}H]-dexamethasone as described previously (*41*). Transfections and luciferase assays were conducted as described by Jones et al. (*42*), using a mouse mammary tumor virus−luciferase reporter plasmid (MMTV−Luc).

RESULTS

Dexamethasone-Mediated Growth Arrest Correlates with the Induction of p21$^{WAF1/Cip1}$. To explore the relationship of

PP5, p21$^{WAF1/Cip1}$, and glucocorticoid mediated growth arrest, we employed three human cell lines (A549, T24, and TR9). Previous studies have shown that the inhibition of PP5 expression with ISIS 15534 inhibits the proliferation of A549 cells with a corresponding increase in the expression of p21$^{WAF1/Cip1}$ (*33*). In contrast, ISIS 15534 does not induce the expression of p21$^{WAF1/Cip1}$ or inhibit proliferation in T-24 and TR9 cells (*33*). Employing the same cell lines under identical culture conditions, dexamethasone also inhibits the proliferation of A549 cells while having no apparent effect on the growth rate of T-24 or TR9 cells (Figure 1). Similarly, following dexamethasone treatment, there is a concomitant increase in the expression of p21$^{WAF1/Cip1}$ in A549 cells that is not observed in T-24 or TR9 cells (Figure 1B). Binding studies with [^{3}H]dexamethasone indicated that T-24 and TR9 cells express GRs (data not shown), and dexamethasone induces the expression of serum/glucocorticoid inducible protein kinase (sgk) in all three cell lines. Thus, the inability of dexamethasone to induce p21$^{WAF1/Cip1}$ expression and growth arrest in T24 and TR9 cells does not appear to result from a lack of functional GRs.

Effect of PP5 on the Binding of Dexamethasone to the GR. Immunoprecipitation studies indicate that PP5 associates with the GR−hsp90 complex (*35*). To determine if PP5 influences hormone binding, binding studies were conducted with [^{3}H]dexamethasone in A549 cells pretreated with ISIS 15534 or a mismatched control (ISIS 15521) analogue of ISIS 15534. The specific binding of [^{3}H]dexamethasone was 77 940 ± 6609, 59 430 ± 9050, and 60 900 ± 6510 cpm/mg of protein in control, ISIS 15534 treated, and ISIS 15521 treated cells, respectively (values are mean ± SE from three independent experiments). Because the inhibition of PP5 expression by treatment with 500 nM ISIS 15534 has no significant effect on the binding of dexamethasone to GRs, it appears that the suppression of PP5 expression does not affect hormone binding.

Comparison of the Effects Induced by Dexamethasone and ISIS 15534 on the Expression of p21$^{WAF1/Cip1}$, PP5, and Serum/Glucocorticoid Inducible Kinase (sgk). Northern analysis indicates that the treatment of logarithmically growing A549 cells with dexamethasone at concentrations of 0.1−100 nM produces a dose-dependent increase in the amount of mRNA encoding both p21$^{WAF1/Cip1}$ and sgk, with maximal expression of both observed following the addition of ∼10 nM dexamethasone (Figure 2). In contrast, dexamethasone treatment has no apparent effect on the level of PP5 mRNA in A549 cells. As reported previously (*33*), treatment with ISIS 15534 produces a dose-dependent decrease in the expression of PP5. This decrease in PP5 mRNA levels correlates with a dose-dependent increase in the expression of p21$^{WAF1/Cip1}$ (Figure 2), and neither the decrease in PP5 mRNA nor the increase in p21$^{WAF1/Cip1}$ mRNA occurs when the cells are treated with mismatched control oligonucleotides [i.e., ISIS 15521; (*33*)]. Treatment with ISIS 15534 also results in a slight increase in sgk mRNA. However, the increase in sgk is less pronounced than that noted for p21$^{WAF1/Cip1}$, and the increase in sgk mRNA produced by 500 nM ISIS 15534 is less than that produced by treatment with 10−100 nM dexamethasone. Interestingly, the effects of dexamethasone and ISIS 15534 are additive for sgk expression (i.e., the dose-dependent increase in sgk expression induced by dexamethasone is greater in A549 cells when the expression of PP5 has been inhibited by previous treatment with 500 nM ISIS 15534).

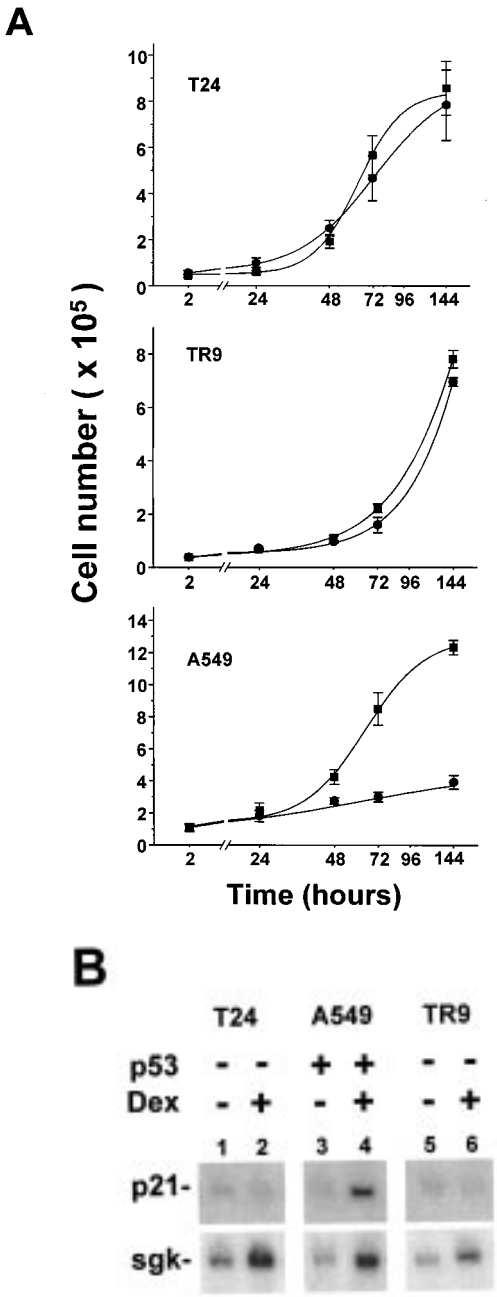

FIGURE 1: Antiproliferative effect of dexamethasone correlates with the presence of wild-type p53 and the expression of p21$^{WAF1/Cip1}$ mRNA. (A) Effect of dexamethasone on cell proliferation in p53-defective T-24 human bladder carcinoma, p53-null TR9 human fibroblasts, and p53 wild-type A549 human lung carcinoma cells was determined by treating cells in log-phase growth with a single dose of 100 nM dexamethasone and counting the number of cells in treated (●) and untreated (■) cultures for 6 consecutive days following treatment. Each point represents the mean of triplicate dishes with error bars representing SE. (B) Comparison of p21$^{WAF1/Cip1}$ (p21) and serum glucocorticoid inducible protein kinase (sgk) mRNA levels in p53-competent (lanes 3, 4) and p53-deficient (lanes 1, 2, 5, 6) cell cultures after dexamethasone treatment. Cell cultures in log-phase growth were treated with 100 nM dexamethasone (+) or vehicle alone as a control (−). After 24 h, total mRNA was prepared and analyzed for p21$^{WAF1/Cip1}$ and sgk mRNA levels by Northern analysis.

Dexamethasone Treatment Is Associated with Enhanced Phosphorylation of p53 in A549 Cells. The ability of both

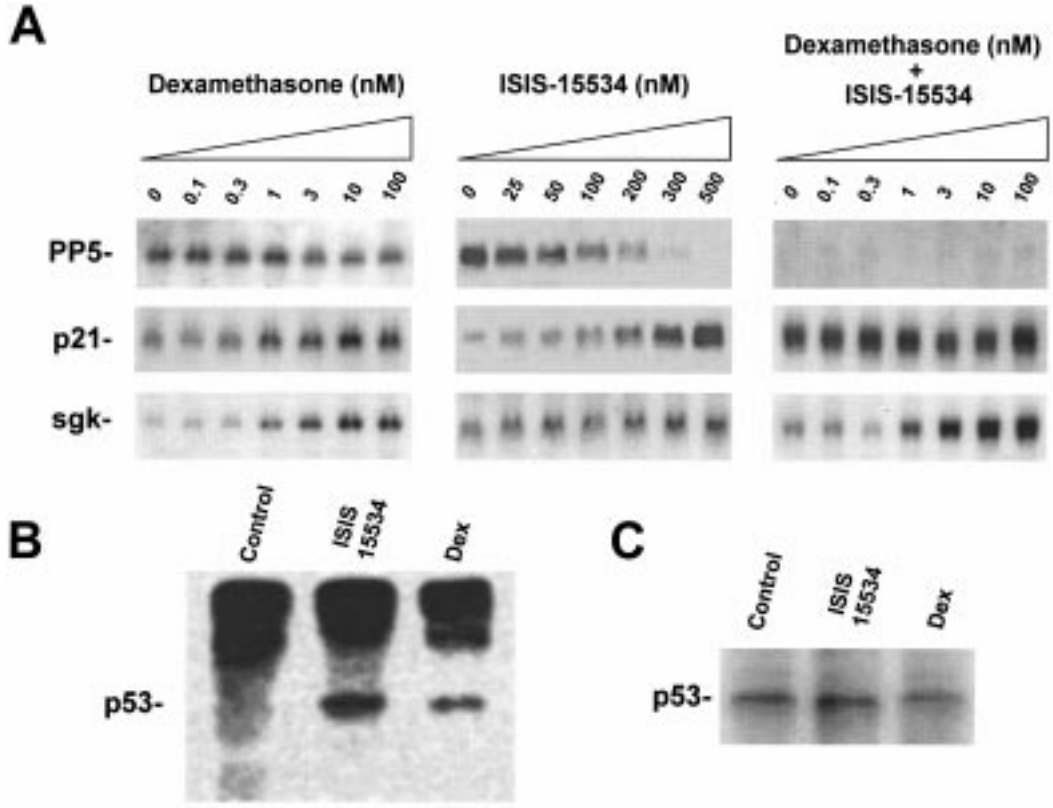

FIGURE 2: (A) Northern blot analysis of A549 cells following treatment with dexamethasone and/or ISIS 15534. Cell cultures in log-phase growth were treated with dexamethasone (0−100 nM), ISIS 15534 (0−500 nM), or the combination of dexamethasone (0−100 nM) and ISIS 15534 (300 nM). After 24 h, 20 μg of total RNA was prepared and analyzed for serine/threonine protein phosphatase 5 (PP5), $p21^{WAF1/Cip1}$ (p21), and serum/glucocorticoid inducible kinase (sgk) mRNA levels. The data shown are representative of three different experiments, and equal loading and transfer of RNA was confirmed by measuring GAPDH mRNA levels (not shown). (B) Hyperphosphorylation of p53 following treatment with ISIS 15534 or dexamethasone. A549 cells were cultured in $[^{32}P]P_i$ and treated with 300 nM ISIS 15534, 100 nM dexamethasone, or vehicle alone (control). Six hours after treatment with dexamethasone, the protein extracts were prepared, and changes in p53 phosphorylation were determined by immunoprecipitation, SDS−PAGE analysis, and visualized by autoradiography. Control, p53 from control cells; ISIS 15534, p53 from cells treated with 300 nM ISIS 15534; Dex, p53 from cells treated with 100 nM dexamethasone. (C) Western analysis of an Immobilon membrane produced by the transfer of protein from the SDS−PAGE gel shown in Figure 2B and detected with an anti-p53 mouse monoclonal antibody.

dexamethasone and ISIS 15534 to induce the expression of $p21^{WAF1/Cip1}$ may reflect the participation of PP5 in a GR-mediated signaling cascade leading to the induction of $p21^{WAF1/Cip1}$ and growth arrest. Alternatively, PP5 and dexamethasone may affect parallel pathways that have a common end point. To explore these two possibilities, we first compared the effects of ISIS 15534 and dexamethasone on several events associated with an increase in the expression of $p21^{WAF1/Cip1}$. In A549 cells, the increase in $p21^{WAF1/Cip1}$ mRNA induced by the inhibition of PP5 expression is dependent on the hyperphosphorylation of preexisting p53, which increases the ability of p53 to bind the p53 consensus response element in the $p21^{WAF1/Cip1}$ promoter (*33*). As seen in Figure 2B,C, treatment of A549 cells with dexamethasone also results in an increase in p53 phosphorylation with no apparent change in p53 protein levels. When considered along with the observation that dexamethasone-mediated induction of sgk is enhanced by the inhibition of PP5 expression (Figure 2), these findings suggest that PP5 affects the expression of GR primary response genes.

To test the ability of PP5 to influence GR-mediated transcription, we first conducted mobility gel-shift studies in A549 cells using ISIS 15534 to inhibit the expression of PP5. As seen in Figure 3, a marked increase in the association of nuclear GR and $[^{32}P]$oligonucleotides corresponding to the consensus glucocorticoid recognition element (GRE) is produced by the inhibition of PP5 expression (Figure 3A; compare lane 2 with lanes 3 and 4). This occurs even in the absence of dexamethasone and indicates that the inhibition of PP5 expression alone is sufficient to enhance the association of GR with GRE. To determine if the increased binding of GR to DNA-containing GRE results in increased transcriptional activity, transient transfection studies were conducted with glucocorticoid responsive reporter plasmids (MMTV−Luc) in A549 cells. As seen in Figure 3B, dexamethasone stimulates transcriptional activity of the GR-reporter plasmid in a dose-dependent manner with the addition of 100 nM dexamethasone producing an ~20-fold increase in luciferase activity. Treatment with ISIS 15534 alone also stimulates the GR-reporter plasmid activity (Figure 3B). However, unlike the gel-shift studies, where a pronounced gel-shift is produced by the inhibition of PP5 expression and only a minor gel-shift is produced by dexamethasone (Figure 3A; compare lanes 4 and 6), GR-reporter plasmid activation is more pronounced after treatment with dexamethasone than after treatment with ISIS 15534 (Figure 3B; compare bar graph with data points).

The inverse relationship, where ISIS 15534 produces a more pronounced increase in the binding of GR to GRE than dexamethasone and dexamethasone induces greater tran-

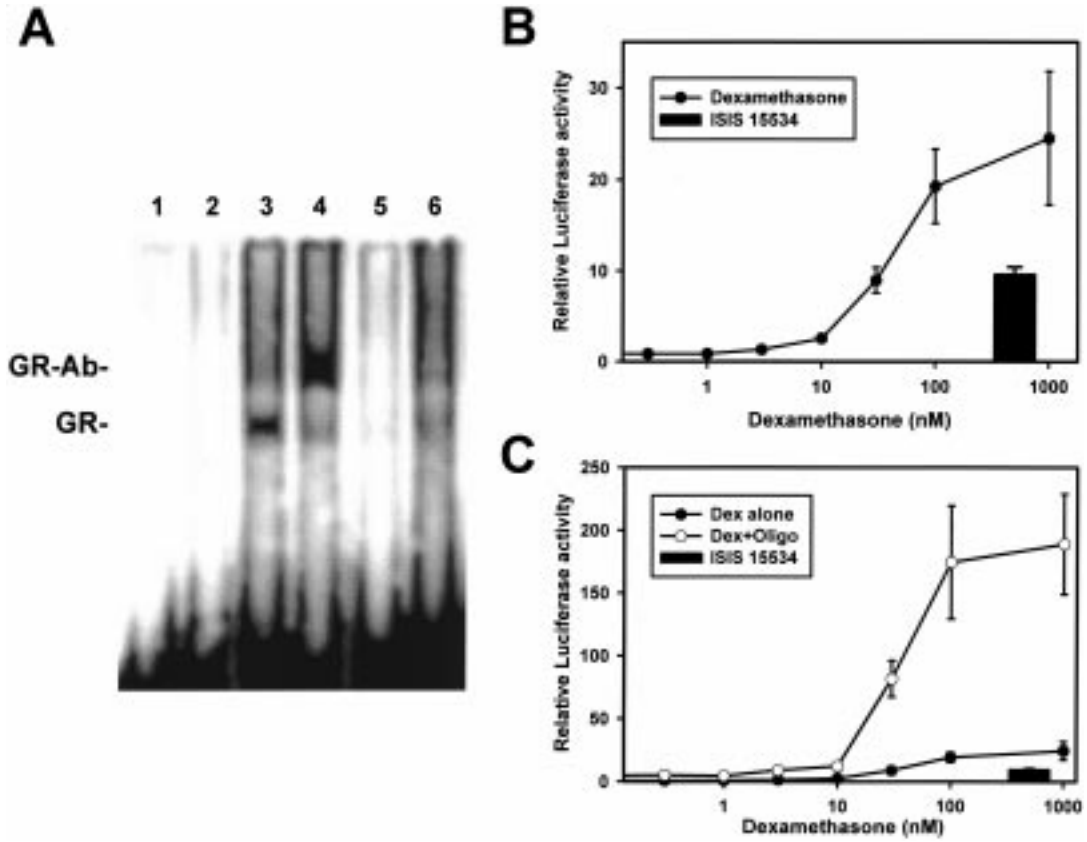

FIGURE 3: (A) Inhibition of PP5 expression enhances glucocorticoid receptor binding to DNA. Nuclear extracts were prepared from A549 cell cultures treated with mismatched control oligodeoxynucleotides (ISIS 15521), ISIS 15534, or 1 μM dexamethasone. After 16 h, the ability of GR to bind DNA was analyzed by gel-mobility shift assay. Lane 1, no protein control: migration of ^{32}P-GRE probe in the absence of nuclear extracts; lane 2, control: nuclear extracts prepared from mismatch control oligodeoxynucleotide treated cells (ISIS 15521; 500 nM); lane 3, ISIS 15534-induced gel shift: nuclear extracts from cells treated with 300 nM ISIS 15534; lane 4, GR antibody-induced supershift: nuclear extracts used in lane 3 after further incubation with an antibody generated against GR; lane 5, excess cold probe control: samples treated in an identical manner as in lane 3 after incubation in the presence of excess nonradioactive GRE probe; lane 6, dexamethasone-induced supershift: nuclear extracts from cells treated with 1 μM dexamethasone following incubation with the GR antibody used in lane 4. (B) Stimulation of GRE promoter activity with dexamethasone or ISIS 15534. A549 cells were transiently transfected with the GR luciferase reporter plasmid MMTV−Luc. Twenty-four hours later, the cells were treated with a single dose of the indicated amount of dexamethasone (●) for 2 h at 37 °C, harvested, washed twice with PBS, and lysed. Aliquots (150 μL) of cell extracts were then assayed for luciferase activity. The relative light units of the cell extract were calculated as an average of three independent experiments, performed in triplicate, and presented as relative luciferase activity with error bars indicating SD. Luciferase activity in cells treated with 500 nM ISIS 15534 in the absence of dexamethasone is indicated by the solid bar. (C) Inhibition of PP5 expression enhances glucocorticoid stimulation of GR reporter plasmids. The transient transfection studies described in panel B were repeated with an additional experimental group (○) in which PP5 mRNA expression was inhibited by treatment with 500 nM ISIS 15534 18 h prior to the addition of dexamethasone. Note the difference in scale on the *y*-axis.

scriptional activity than ISIS 15534, suggests that the inhibition of PP5 expression enhances the association of GR with GRE, but maximal activation of the GR−transcriptional complex requires glucocorticoids. To test this, we conducted transient transfection studies with GR-reporter plasmids in A549 cells 24 h after the expression of PP5 was inhibited with ISIS 15534. The combined treatment resulted in a dose-dependent increase in GR-reporter plasmid activation, producing a maximal increase in luciferase activity that is ~200-fold above the control and ~10 times greater than the maximum response achieved with dexamethasone alone (Figure 3). Gel-shift analysis following combined treatment did not reveal an apparent increase in GR/GRE interactions beyond that produced by treatment with ISIS 15534 alone (data not shown), and transient transfection studies conducted with glucocorticoid-responsive reporter complexes in T-24 and TR9 cells revealed that treatment with dexamethasone alone, ISIS 15534 alone, or the combination of ISIS 15534 and then dexamethasone induces reporter activity in a manner similar to that observed in A549 cells (dexamethasone + ISIS 15534 ≫ dexamethasone > ISIS 15534).

DISCUSSION

The growth and differentiation of cells is controlled by complex regulatory events that must be coordinated to meet the demands of cells, tissues, and ultimately the organism as a whole. In mammals, the fundamental decision of a cell to either replicate its DNA and divide or withdraw from the cell cycle with an unduplicated genome appears to take place in mid-to-late G1-phase of the cell cycle, and the commitment to either of these pathways reflects the integration and processing of information derived from numerous positive and negative signaling cascades. The data presented here suggest that PP5 is a key regulator of cellular proliferation in A549 cells, affecting a G1-checkpoint control mechanism utilized by both steroid hormone- and stress-induced signaling networks to impede or inhibit progression through the

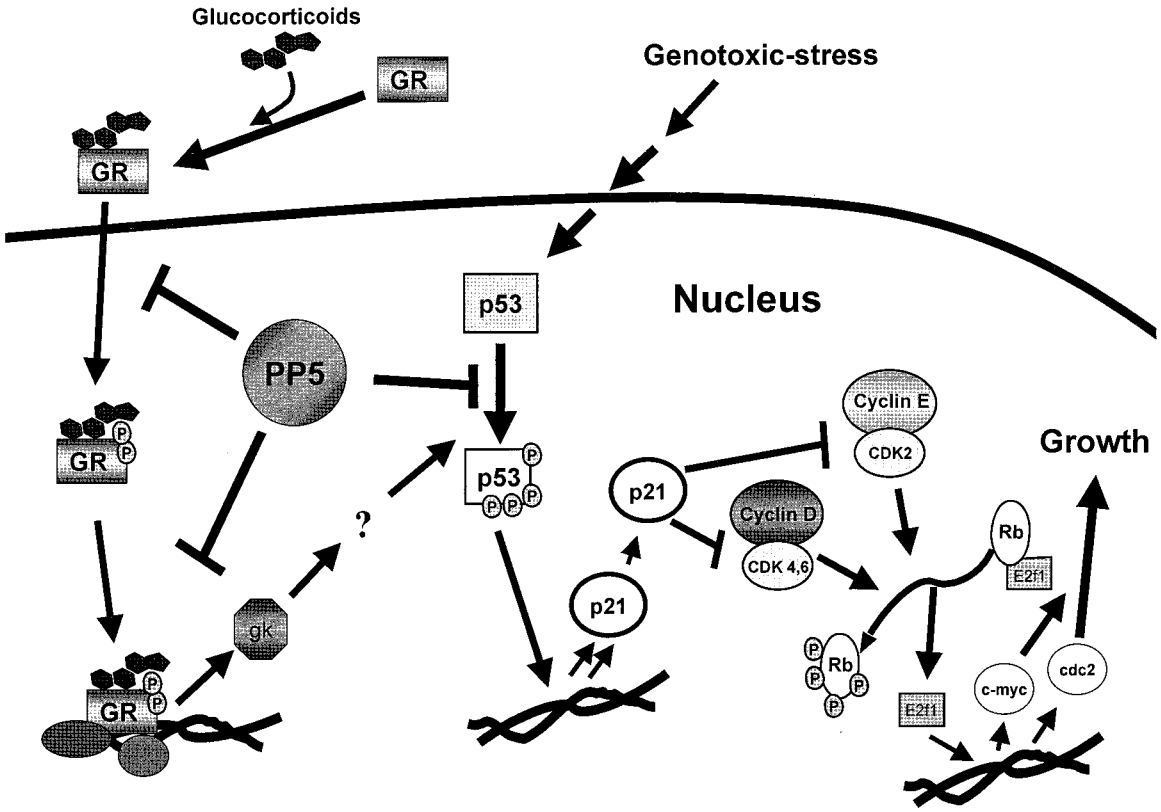

FIGURE 4: Proposed role of PP5 in the regulation of G1-growth arrest initiated by glucocorticoids or genotoxic stress. The increase in PP5 expression that occurs during log-phase cell growth functions to inhibit glucocorticoid-mediated growth arrest by blocking the association of the ligand-activated GR complex with specific recognition sequences in the promoter of glucocorticoid-responsive genes and/or the formation of an active GR−transcriptional complex. This inhibits the expression of a GR-inducible protein kinase (gk) or a signal transduction cascade that results in the activation of a kinase that catalyzes the phosphorylation of p53. In the absence of PP5, the hyperphosphorylated form of p53 produced has increased transcriptional activity, which allows the basal amount of p53 to induce the expression of the cyclin/CDK inhibitor protein, $p21^{WAF1/Cip1}$ (p21). $p21^{WAF1/Cip1}$ inhibits the activity of G1−cyclin/CDK complexes (Cyclin D/CKD 4,6; Cyclin E/CDK2). In turn, this prevents the hyperphosphorylation of the Rb-tumor suppressor protein and facilitates G1 cell cycle arrest. In response to genotoxic stress, the amount of p53 increases due to a decrease in degradation rate and, in some instances, an increase in p53 expression (*51*−*58*). In the presence of PP5, the increased amount of p53 may be necessary to "over ride" the growth-promoting effects of PP5.

G1/S-phase of the cell cycle via the transcriptional regulation of $p21^{WAF1/Cip1}$.

The hypothesis that PP5 affects GR-induced growth suppression arose from previous studies showing that the expression of $p21^{WAF1/Cip1}$ is induced following treatment with either ISIS 15534 or dexamethasone (*7*−*9*, *33*) and the observation that PP5 coimmunoprecipitates with the GR−hsp90 complex (*34*, *35*). Review of the literature revealed that reversible Ser/Thr phosphorylation may influence (1) the binding of hormone to the cytoplasmic GR receptor complex, (2) the translocation of the GR into the nucleus, (3) the binding of activated GR to consensus GRE in the promoter regions of GR-responsive genes, and (4) the formation of an active transcriptional complex (*1*−*6*, *43*−*50*). To explore the role of PP5 in these processes, we first compared the ability of [^{3}H]dexamethasone to bind GR in the presence and absence of PP5. These studies indicated that the suppression of PP5 expression has no apparent effect on the binding of dexamethasone to the GR, suggesting that PP5 does not affect the binding of hormone to the GR. In contrast, when PP5 expression was suppressed, gel-shift analysis revealed a marked increase in the amount of GR associated with DNA containing consensus GREs, and GR-reporter plasmids were activated without the addition of dexamethasone. In addition, the combined treatment of ISIS 15534 and dexamethasone induced reporter plasmid activation to a level that is ∼10 times greater than the maximal response attainable in the presence of PP5. Reversible phosphorylation has been shown to influence the association of GR with GRE, and several Ser/Thr residues in the transcriptional regulatory domain of the GR either positively or negatively regulate GR transcriptional activation (*43*−*48*). Because treatment with ISIS 15534 alone produces a gel-shift that is much greater than the maximal shift produced by dexamethasone, yet ∼50% less transcription of the GR-reporter plasmid, the current data suggest that PP5 functions to regulate GR /GRE interactions or the nuclear accumulation of GR, with hormone binding regulating the formation of a fully active complex. Unfortunately, we are unable to directly assess the ability of PP5 to regulate the phosphorylation of GR, because ISIS 15534 targets a region of human PP5 that is not sufficiently conserved in rodents and antibodies that efficiently immunoprecipitate GR in human cells are not available.

The relationship of PP5, GR, p53, and $p21^{WAF1/Cip1}$ is complex. The induction of $p21^{WAF1/Cip1}$ by p53 occurs in response to the direct interaction of p53 with its cognate binding site in the $p21^{WAF1/Cip1}$ promoter (for review, see ref *51*), and it is established that the p53 protein functions as a key regulator of $p21^{WAF1/Cip1}$ expression in response to stress-induced signaling cascades initiated by DNA damage (*52*−*54*), hypoxia (*55*), or nucleotide deprivation (*56*). There are

two major aspects of p53 transactivation of $p21^{WAF1/Cip1}$: accumulation and activation (*57*, *58*). Nuclear accumulation of p53 is caused primarily by an increase in the half-life of the p53 protein, and the accumulation of p53 is associated with the transactivation of p53-responsive genes (*51*, *52*). However, the transactivation by p53 of genes required for cell cycle arrest does not necessarily require an increase in the amount of p53 protein (*33*, *57*−*59*), and recent studies indicate that both the stabilization and the activation of p53 are regulated independently by different phosphorylation events (*52*).

GR activation of $p21^{WAF1/Cip1}$ expression is less well understood. GR-induced $p21^{WAF1/Cip1}$ expression has been proposed to occur via direct protein−protein interactions of the activated GR with preexisting $p21^{WAF1/Cip1}$ promoter-bound transcription factors (*9*) and, in some cells of hepatic origin, by the induction of C/EBPα, which subsequently stimulates $p21^{WAF1/Cip1}$ promoter activity by direct binding to cognate DNA binding sites in the $p21^{WAF1/Cip1}$ promoter (*22*). Our studies revealed that dexamethasone-mediated induction of $p21^{WAF1/Cip1}$ is associated with an increase in p53 phosphorylation, suggesting that p53 may participate in the GR-induced signaling networks that control $p21^{WAF1/Cip1}$ expression.

To study the relationship of PP5, p53, and GR in the regulation of $p21^{WAF1/Cip1}$ expression, we employed A549 cells, which are growth arrested by treatment with dexamethasone and wild type for p53. In A549 cells, both ISIS 15534- and dexamethasone-induced growth inhibition occurs with a concomitant increase in the expression of $p21^{WAF1/Cip1}$. In contrast, neither ISIS 15534 nor dexamethasone inhibits proliferation or induces $p21^{WAF1/Cip1}$ expression in p53-defective or -deficient cell lines (i.e., T-24 or TR9 cells). Having convincing data that PP5 affects both GR- and p53 -mediated induction of $p21^{WAF1/Cip1}$, we wanted to determine if (1) PP5 affects two separate signaling pathways leading to the induction of $p21^{WAF1/Cip1}$ and growth arrest (one mediated by p53 and the other mediated by GR activation) or (2) if PP5 affects one pathway in which GR-mediated induction of $p21^{WAF1/Cip1}$ occurs via a p53-dependent process. Binding studies, Northern analysis, and transient transfection studies with glucocorticoid-responsive reporter plasmids indicate that A549, T-24, and TR9 cell lines all express functional GRs. Thus, the inability of dexamethasone to induce $p21^{WAF1/Cip1}$ expression in T-24 and TR9 cells does not appear to result from the lack of functional GRs. To explore events downstream of GR-induced transcription, we employed TR9-7 cells, a stable cell line derived from $p53^{-/-}$ human fibroblasts into which wild-type p53 was reintroduced under the control of tetracycline-regulated transactivator and operator plasmids (*36*). In TR9-7 cells, the expression of p53 at high levels is sufficient to induce the expression of $p21^{WAF1/Cip1}$ and induce growth arrest without any additional stimuli (*36*), and neither the inhibition of PP5 expression nor the addition of dexamethasone inhibits cell growth or induces the expression of $p21^{WAF1/Cip1}$ when the expression of wild-type p53 is inhibited by the presence of 2 μM tetracycline (*33*, Figure 1). However, when wild-type p53 is reintroduced at a low level, (i.e., a level comparable to the basal amount of p53 expressed in nonstressed fibroblasts that does not induce $p21^{WAF1/Cip1}$ expression on its own), the hyperphosphorylation of p53 produced by ISIS 15534-mediated inhibition of PP5 expression is sufficient to induce $p21^{WAF1/Cip1}$ expression and growth-arrest (*33*). Thus, PP5 functions as an inhibitor of phosphorylation-dependent p53 activation (*33*). As observed following treatment with ISIS 15534, dexamethasone-mediated induction of $p21^{WAF1/Cip1}$ expression in A549 cells is also associated with an increase in the phosphorylation of p53 (Figure 3). This supports the concept that the phosphorylation-dependent activation of p53 arises from a signaling cascade initiated by glucocorticoids. The role of p53 in GR-mediated induction of $p21^{WAF1/Cip1}$ is also supported by the lack of a single report in the literature that demonstrates GR-mediated growth arrest in p53-deficient cell lines, and the lack of a GRE in the p53 promoter argues against the direct induction of p53 by hormone-activated GR. Still, we were unable to restore dexamethasone-mediated growth arrest in TR9-7 cells by expressing low levels of p53 (data not shown). This failure could argue for a separate GR-mediated signaling cascade leading to the induction of $p21^{WAF1/Cip1}$. Alternatively, the inhibitory effect of PP5 may dominate over the stimulatory effect of dexamethasone (i.e., in the presence of PP5, dexamethasone-induced phosphorylation of p53 is not sufficient to overcome the countering effects of PP5). If the latter proves true, both p53 accumulation and increased p53 phosphorylation may be necessary for the induction of $p21^{WAF1/Cip1}$ expression when PP5 expression is high. Unfortunately, further testing of hypotheses designed to determine the precise relationship of PP5, p53, and $p21^{WAF1/Cip1}$ has proven difficult because either the inhibition of PP5 expression or the accumulation of p53 alone induces $p21^{WAF1/Cip1}$ expression and growth arrest in A549 cells.

From the data presented here and those available in the literature, we propose that PP5 functions as a promoter of cellular proliferation by inhibiting both glucocorticoid and DNA damage/stress-induced signaling cascades that control the expression of $p21^{WAF1/Cip1}$ (Figure 4). In addition to the well-established role in the propagation of a growth inhibitory response initiated by stress (*51*), our data suggest that the p53 tumor suppressor protein may also directly participate in a GR-induced signaling network leading to G1 growth, with the basal expression of p53 facilitating the propagation of a GR-induced antiproliferative response. This antiproliferative response is countered by PP5, and in the absence of PP5, the ensuing increase in GR-induced transcriptional activity together with the concomitant hyperphosphorylation of p53 increases the transcription of $p21^{WAF1/Cip1}$ in some cells (*33*). Thus, the increased expression of PP5 observed during log-phase cell growth may function to facilitate cellular proliferation by preventing the transactivation of genes by both GR and p53. However, although the data suggest that p53 may be necessary for glucocorticoid-mediated G1-growth arrest in A549 cells, they do not exclude the existence of additional GR-induced mechanisms leading to the induction of $p21^{WAF1/Cip1}$.

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