Prostaglandin A2 Blocks the Activation of G1 Phase Cyclin-dependent Kinase without Altering Mitogen-activated Protein Kinase Stimulation*

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Prostaglandin A2 (PGA2) reversibly blocked the cell cycle progression of NIH 3T3 cells at G1 and G2/M phase. When it was applied to cells synchronized in G0 or S phase, cells were blocked at G1 and G2/M, respectively. The G2/M blockage was transient. Microinjected oncogenic leucine 61 Ras protein could not override the PGA2-induced G1 blockage, nor could previous transformation with the v-raf oncogene. The serum-induced activation of mitogen-activated protein kinase was not inhibited by PGA2 treatment. These data suggest that PGA2 blocks cell cycle progression without interfering with the cytosolic proliferative signaling pathway. Combined microinjection of E2F-1 and DP-1 proteins or microinjected adenovirus E1A protein, however, could induce S phase in cells arrested in G1 by PGA2, indicating that PGA2 does not directly inhibit the process of DNA synthesis. In quiescent cells, PGA2 blocked the normal hyperphosphorylation of the retinoblastoma susceptible gene product and the activation of cyclin-dependent kinase (CDK) 2 and CDK4, in response to serum stimulation. PGA2 treatment elevated the p21Waf1/Cip1/Sdi1 protein expression level. These data indicate that PGA2 may arrest the cell cycle in G1 by interfering with the activation of G1 phase CDKs.

For cell cycle transition from G0/G1 to S phase, at least three types of molecular systems are involved in a concerted manner. These include: 1) the signal transduction pathway which receives extracellular signals and transmits these into the cell, 2) cyclins with associated kinases and modulators which may regulate passage through the G1 restriction point and other cell cycle check points, and 3) the metabolic processes required for doubling the essential cellular components including DNA.

While a variety of signaling systems work together to either induce or block proliferation, one of the best characterized, and perhaps the most universally required signaling systems for proliferation, involves proto-oncogenes including cellular Ras proteins. When growth factors bind to their tyrosine kinase receptors, a series of phosphorylations and resulting intramolecular interactions induce the activation of cellular Ras proteins (1). Active Ras in turn binds to cellular Raf kinases resulting in their activation and ultimately the activation of mitogen-activated protein kinases (MAP kinases) (2). The activated MAP kinases enter the nucleus and presumably stimulate the activity of genes required for proliferation (3, 4).

Even though cellular proliferation in most cell types requires the activity of the above signal transduction system, the orderly transit of the growth factor-stimulated cell through the cell cycle depends upon the action of the second class of nuclear proteins, including cyclins, CDKs, and proteins which modulate the activity of the complexes they form. It is believed that cyclins and associated CDKs control progress through cell cycle phases. In so doing they would regulate the activity of the third group of molecules required for cell proliferation, those which catalyze the metabolism required to duplicate DNA and other critical cellular components necessary for cell division. For example, active cyclin D/CDK4 is known to phosphorylate the pRb (5, 6). Hyperphosphorylation of the pRb results in the release of the transcription factor complex E2F/DP which is bound to and inactivated by hypophosphorylated pRb (7). Active E2F/DP is known to induce the transcription of molecules required for DNA synthesis, such as dihydrofolate reductase (8).

A full understanding of the control of cell cycle progression from G0 to S phase will require not only an understanding of these three separate processes essential for cell cycle progression (signaling molecules, cyclins and associated proteins, and enzymes required for DNA synthesis), but an understanding of how these classes of proteins interact with each other. As described above and elsewhere (9), the molecular mechanism connecting the activity of cyclins and their associated proteins to DNA synthesis is well characterized. Of particular interest in this study is the interaction between proto-oncogene signaling molecules and cyclin-associated proteins. Direct evidence for such an interaction was obtained in a recent microinjection study. The proliferation of most normal cells is blocked following the microinjection of a neutralizing anti-Ras antibody (10). When cells which have received such injections receive a subsequent injection of purified adenoaviral E1A protein which is able to release E2F/DP from pRb (7, 11), the cells are able to rapidly enter S phase with high efficiency (12). Thus, the activity of E2F/DP, a normal consequence of cyclin-associated protein action, is able to compensate for blockage of the action of proto-oncogene signaling molecules. It therefore appears that Ras activity in the cell is required for entry into S phase to

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1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase; CDK, cyclin-dependent kinase; pRb, product of retinoblastoma susceptible gene; PGA2, prostaglandin A2; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TGF-β, transforming growth factor β.
Prostaglandin A 2 Inhibits Cyclin-dependent Kinase

Materials—Recombinant truncated human pRB was supplied from ImmunoPharmaquecs Inc. (San Diego, CA). Anti-pRB antibody (mouse monoclonal, PMG3-245) was a product of Pharmingen (San Diego, CA). Anti-ERK2 (rabbit polyclonal, C-14), anti-CDK2 (rabbit polyclonal, M2), anti-CDK4 (rabbit polyclonal, C-22), anti-cyclin E (rabbit polyclonal, M-20), anti-cyclin D1 (mouse monoclonal, 72-13G), and anti-p21Waf1/Cip1/Sdi1 (goat polyclonal, C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ATP, rabbit anti-mouse IgG, and bovine myelin basic protein were obtained from Sigma. Histone H1 and horseradish peroxidase conjugate of anti-goat IgG and anti-mouse IgG+IgM were supplied from Boehringer Mannheim. Protein A-Sepharose was a product of Zymed Laboratories Inc. (South San Francisco, CA). PGA2 was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Autoradiography emulsion type NTB2 was a product of Eastman Kodak Co. (New York). Western blot chemiluminescence reagent and Reflection™ autoradiography film were obtained from Amersham. [3H]thymidine (80 Ci/mmol) was a product of Amersham. [γ-32P]ATP (3,000 Ci/mmol) was purchased from ICN.

Cell Culture—NIH 3T3 cells and v-raf transformed NIH 3T3 cells (16) were maintained in 10% calf serum, 100 units/ml penicillin- and streptomycin-supplemented Dulbecco’s modified Eagle’s essential medium. The subconfluent NIH 3T3 cells were made quiescent by serum starvation (0.5% calf serum) for 36 to 48 h. Serum-starved NIH 3T3 cells (16) were supplied from Boehringer Mannheim. Protein A-Sepharose was a product of Zymed Laboratories Inc. (San Francisco, CA). PGA2 was purchased from Biomed Research Laboratories, Inc. (Plymouth Meeting, PA).

Preparation and Microinjection of Oncoproteins—Leu-61 Ras, E1A, E2F-1, and DP-1 proteins were prepared as described (12, 18). Cells were grown on coverslips. All the cells inside the circle (approximately 100–200 cells), which was drawn on the non-cell-growing side of coverslips, were injected with protein(s). After 20–24 h of labeling with [3H]thymidine and autoradiography, the labeling indices were determined for the cells inside the circle (injected cells) and outside the circle (noninjected cells).

RESULTS

To determine an inhibitory range of PGA2, rapidly growing cultures of NIH 3T3 cells were treated with various concentrations for 24 h, labeled with [3H]thymidine for 4 h at the end of the PGA2 treatment, fixed, and autoradiographed. Parallel cultures were treated similarly except that, after 24 h, the PGA2 was removed and replaced with normal medium containing [3H]thymidine for an additional 24 h, to determine reversibility. Concentrations of PGA2 higher than 20 μM efficiently blocked thymidine incorporation, while concentrations of 25 μM or less were found to be reversible (Fig. 1A). Reversible inhibition was observed even after 48 h of PGA2 treatment, although continued inhibition required addition of fresh PGA2 every 24 h (data not shown). Concentrations above 35 μM were cytotoxic.

Cell Cycle Considerations—A thorough analysis of the cell

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cycle consequences of the action of PGA2 upon NIH 3T3 cells was undertaken to determine precisely where in the cell cycle treated cells were blocked. Rapidly growing cells were treated with PGA2 and labeled with [3H]thymidine for a period of 1 h at various times thereafter. As a control, parallel cultures were treated with 1 mM hydroxyurea, which is known to block DNA synthesis by depleting the deoxyribonucleotide pool (27). In the hydroxyurea-treated cultures, thymidine labeling was reduced within 1 h and blocked within 2 h of treatment. In the PGA2-treated cultures, on the other hand, thymidine labeling began to be reduced only after 5 h of treatment (Fig. 1B). This result clearly indicates that, unlike hydroxyurea, PGA2 does not block the progress of an ongoing cycle of DNA synthesis and, therefore, does not interfere with the enzymes required for DNA synthesis.

To further analyze the point at which PGA2 inhibits cell cycle progression, asynchronously growing cultures were treated with PGA2 for 24 h, and their DNA content was analyzed by flow cytometry. In accordance with previously reported data, it was apparent that blockage at multiple points takes place (28). Such cultures exhibited an increased proportion of the cells in both G1 and G2/M phases, while the number of the cells in S phase was reduced (Fig. 2, A and B).

In order to confirm that cell cycle blockage can take place in either G1 or G2/M phase, synchronized cultures were treated with PGA2. When the culture was synchronized in G0 by serum deprivation (Fig. 2C) prior to treatment with PGA2 and stimulation with serum, essentially all the cells were blocked in G0, as indicated by their DNA content (Fig. 2E). Control cultures stimulated with serum in the absence of PGA2 progressed into S phase (Fig. 2D). Cultures treated with PGA2 at 15 h after serum addition, when most cells were in S phase (Fig. 2D), appeared in G0/M phase after 24 h (Fig. 2G). The fact that most of the S phase cells progressed into G0/M phase in the presence of PGA2 confirms the previous results indicating that PGA2 does not block the progress of an ongoing S phase. The fact that few of these cells had progressed through mitosis into G1, as would have been the case in the absence of PGA2 (Fig. 2F), indicated the presence of a G0/M phase block by the inhibitor. There was, however, a clear distinction between G0 and G2/M blockages. While the G1 arrest could be maintained for 48 h by replenishing the PGA2-containing medium every 24 h, the majority of G2/M arrested cells, in the presence of PGA2, eventually went through the mitosis and were blocked in G1 at 20 h after the addition of PGA2 to synchronous S phase cells (data not shown). This indicates that PGA2-induced G2/M blockage is transient or that PGA2 treatment results in the elongation of time required for progression through G2/M phases. The blockage at two different cell cycle points may indicate that PGA2 targets a molecule(s) important for the regulation of cell cycle progression.
PGA2 was efficiently able to block DNA synthesis, the target of PGA2 inhibition. In these studies, biochemical and biological markers of both proliferative signaling and cell cycle regulation systems were analyzed. To begin with, the injection of PGA2 into cultured NIH 3T3 cells transformed by oncogenic Ha-Ras mutant Leu-61 expressed in bacterial cells is able to function efficiently following microinjection into cultured cells. In NIH 3T3 cells, the injection of this protein efficiently induced morphologic transformation and cell cycle progression even in the absence of serum (29). If PGA2 blocks proliferative signal transduction by inhibiting the activity of a molecule required for the activation of cellular Ras, it is expected that the introduction of oncogenic Ras protein would override this inhibition and allow continued cell cycle progression even in the presence of PGA2. Such an observation was made in TGF-β-treated cells, suggesting that TGF-β functions upstream of cellular Ras (30). With PGA2, however, the opposite result was obtained. While the injected Leu-61 Ras was able to induce effective entry into S phase when injected into serum-deprived NIH 3T3 cells, it was unable to induce any noticeable thymidine labeling above background in PGA2-treated cells (Fig. 4). These results suggest that PGA2 targets a function downstream of cellular Ras activity and downstream of the target of TGF-β.

To confirm the observation that PGA2 interferes with proliferative signaling at a point subsequent to the action of cellular Ras, the action of two well characterized downstream targets of Ras activity were analyzed. The effect of PGA2 upon v-raf-transformed cells was first examined. A number of biological and biochemical observations confirm that biologically active Ras proteins induce the activation of cellular Raf proteins. When PGA2 was added to NIH 3T3 cells transformed by oncogenic raf, thymidine incorporation was blocked in a dose-dependent and reversible manner (Fig. 1A). In addition, when raf-transformed cells were treated with PGA2 and subjected to FACS analysis 24 h later, the cells were seen to be inhibited in G1 and G2/M (Fig. 2, H and I), as was observed with nontransformed NIH 3T3 cells. The fact that raf-transformed cells were effectively inhibited in cell cycle progression by PGA2 is consistent with the idea that PGA2 inhibits a molecule whose function is required subsequently to the action of cellular Raf protein.

The biological studies were extended with biochemical analysis of MAP kinase, a molecule whose activity is stimulated by both Ras and Raf activity, and which therefore functions down-
stream of both in proliferative signaling. The activity of MAP kinase was assessed by immunoprecipitation with specific anti-Erk2 antibodies followed by incubation with a substrate, myelin basic protein, in the presence of \( \gamma^32P \) ATP. The amount of the myelin basic protein phosphorylation indicates the level of MAP kinase activity in the cell at the time of immunoprecipitation. Addition of serum to serum-deprived cells results in a rapid, more than 10- to 20-fold, increase in the activity of MAP kinase compared to quiescent cells (Fig. 5, lanes 2 and 3). When PGA2 was added together with serum or when the cells were pretreated with PGA2 for 2 h prior to the addition of medium containing serum and PGA2, the activation of MAP kinase was not altered (Fig. 5, lanes 4 and 5). PGA2 itself has little effect on the basal MAP kinase activity (Fig. 5, lanes 1 and 2). The fact that neither injected Leu-61 Ras nor transformation with oncogenic raf! can interfere with PGA2 inhibition, together with the fact that serum-induced activation of MAP kinase is not altered by PGA2 treatment, clearly indicates that it blocks an activity which is essential to proliferation but which is not apparently involved in the action of several well characterized proto-oncogene components of the proliferative signal transduction pathway.

Microinjection of Adenoviral E1A or E2F-1/DP-1 Transcription Factors—Based upon the above considerations, we conclude that PGA2 is able to efficiently block cell cycle progression late in G1, without interfering with the activation of several molecules (Ras, Raf, MAP kinase) known to function in the proto-oncogene signaling pathway. It was, therefore, considered to be possible that PGA2 either targets the action of cyclins and related proteins, or it might be interfering with a metabolic process required for S phase. Although PGA2 did not inhibit ongoing DNA synthesis (Fig. 1B), this assay may not be sensitive enough to detect the inhibition of a very early step during the initiation of DNA synthesis. To test these two possibilities, other biological markers of proliferative control were utilized, the E2F-1 transcription factor and the E1A adenoviral protein. E2F-1 is known to activate the transcription of a set of genes whose activities are required for DNA synthesis. Consequently, microinjected E2F-1 can induce S phase in the cells which are arrested in G1 by previous injection of anti-Ras antibody. Adenoviral E1A protein is known to interact with a number of cell cycle regulatory proteins including the pRb and, through this interaction, E1A protein releases the active E2F transcription factor. Because injected E1A is also able to induce thymidine labeling even in anti-Ras containing cells (31), E2F-1 and E1A serve as biological markers of proliferative control downstream of Ras.

Although E2F-1 microinjection induced S phase in serum-starved cells, it failed to efficiently override the PGA2 G1 arrest (Fig. 4). Since DP-1 is another transcription factor which associates with E2F-1 and potentiates the E2F transcription activity (7), we tested the S phase inducing activity of DP-1 protein. While microinjection of DP-1 alone did not induce S phase in serum-starved cells, it also failed to override PGA2 blockage. Combined microinjection of E2F-1 and DP-1, however, was able to override the PGA2 growth arrest efficiently. As a biological marker of proliferative control which is somewhat upstream of E2F/DP-1 activity, we next microinjected E1A protein. When purified E1A protein was microinjected into NIH 3T3 cells treated with PGA2, the cells were efficiently induced to incorporate labeled thymidine (Fig. 4). Because E2F-1/DP-1 and the modulation of the activity of the molecules targeted by E1A were able to overcome PGA2 inhibition, this inhibition must involve molecules required for the control of proliferation rather than a metabolic process required for DNA synthesis. Furthermore, it is clear that PGA2 inhibits a step involved in cell cycle control which functions prior to the action of the targets of E1A and the release of E2F/DP-1 transcription factor.

Blockage of pRb Hyperphosphorylation and Cyclin/CDK Activation—To more carefully characterize the target of PGA2 action, additional biochemical markers were analyzed. NIH 3T3 cells were rendered quiescent by serum deprivation for 48 h. Serum was then added to these cultures with or without PGA2 for 12 h prior to harvesting the cells. The pRb was then immunoprecipitated and subjected to Western blot analysis. Cells stimulated mitogenically respond by phosphorylating pRb and thereby activating E2F activity. The hyperphosphorylated pRb has a reduced mobility in SDS-PAGE (20). In cultures treated with serum alone, the pRb exhibited this mobility shift, while, in the presence of PGA2, no such shift was apparent (Fig. 6). This indicates that while PGA2 does not apparently interfere with the action of proto-oncogenes, it does interfere with the process of proliferative control prior to the hyperphosphorylation of pRb.

There is extensive evidence that pRb phosphorylation requires the activation of cyclins and their associated kinases. Cyclin D/CDK4 becomes an active kinase in mid to late G1 and has the ability to phosphorylate pRb directly (32). Later in G1, apparently near the restriction point, cyclin E/CDK2 becomes active. The effect of PGA2 upon these two kinases was, therefore, analyzed. NIH 3T3 cells were deprived of serum for 48 h prior to the addition of serum alone, or serum and PGA2 together. These cells were cultured for an additional 10 h and
ical studies suggest that PGA2 is taken up by a carrier system. Serumin CDK4 immunoprecipitate. In the presence of PGA2, however, phosphorylate pRb as indicated either in the cyclin D or in the serum stimulated the ability of the cyclin D/CDK4 complex to activity of both kinases was low in quiescent cells. The added PGAs were separated by SDS-PAGE and autoradiographed. The kinase activity associating with the precipitate was determined using recombinant pRb (for CDk4 and cyclin D1) or histone H1 (for CDK2 and cyclin E). The result was reproducible in 2 (for CDK4, cyclin D1, and cyclin E immunoprecipitation) or in 4 (for CDK2 immunoprecipitation) separate experiments.

lysates were prepared from these lysates, immunoprecipitates were made with antibodies against CDK2, CDK4, cyclin D, or cyclin E. The immunoprecipitates were then incubated with the appropriate substrate to detect kinase activity; recombinant pRb in the case of CDK4 and cyclin D or histone H1 in the case of CDK2 and cyclin E. The phosphorylated substrates were then separated by SDS-PAGE and autoradiographed. The activity of both kinases was low in quiescent cells. The added serum stimulated the ability of the cyclin D/CDK4 complex to phosphorlyate pRb as indicated either in the cyclin D or in the CDK4 immunoprecipitate. In the presence of PGA2, however, the kinase activity in each immunoprecipitate was equal to or even less than that seen in quiescent cells (Fig. 7A). In addition, the activity of the cyclin E/CDK2 complex was inhibited by added PGA2. As above, the added serum greatly stimulated the activity of this complex as assessed in immunoprecipitates with either cyclin E or CDK2, while no stimulation was seen in cells treated with serum and PGA2 together (Fig. 7B).

Expression of p21Waf1/Cip1/Sdi1 Protein—As a possible mechanism of G1 phase cyclin-CDK inhibition, we examined the expression level of p21Waf1/Cip1/Sdi1 protein, a CK inhibitor protein (33) which has been shown to mediate G1 growth arrest (25, 34). By Western blotting, the p21Waf1/Cip1/Sdi1 protein level was low in growing cell (Fig. 8A, lanes 1 and 3). Upon PGA2 treatment, p21Waf1/Cip1/Sdi1 protein was induced (Fig. 8A, lane 2). As a positive control, we treated the cells with 5 gray of γ-irradiation, a treatment known to induce p21Waf1/Cip1/Sdi1 protein (Fig. 8A, lane 4). When serum-starved cells received PGA2 together with serum, p21Waf1/Cip1/Sdi1 protein was again induced (Fig. 8B, lanes 6 and 8) compared with quiescent cells treated only with serum (Fig. 8B, lanes 5 and 7). These results indicate that one of the possible mechanisms of CDK inhibition is the induction of p21Waf1/Cip1/Sdi1 protein.

**DISCUSSION**

Although its mechanism of action is unknown, pharmacological studies suggest that PGA2 is taken up by a carrier system at the plasma membrane. After internalization, PGA2 binds to cytosolic proteins and moves to the nucleus (35). Cell cycle blockage in mid to late G1 has been well documented (36). In this study, the blockage by PGA2 in NIH 3T3 cells was confirmed to be in late G1, within 3 h of the initiation of DNA synthesis. This observation suggests that PGA2 might be interacting with a molecule (molecules) critical for passage through the restriction point just prior to S phase. In addition, a blockage in G2/M is also observed as reported previously (28). To confirm these results, cells were synchronized in either G0 or S phase at the time of PGA2 addition. In such cultures, cells became blocked either in G1 or in G2/M, respectively, although blockage in G2/M was transient. No evidence for blockage in S phase was obtained. It, therefore, appears likely that PGA2 targets a molecule(s) required for cell cycle progression both in late G1 and in G2/M phase.

Analyses were performed to localize PGA2 inhibition in relationship to well characterized molecules involved in cell proliferation. The fact that microinjected oncogenic Ras was unable to overcome the inhibitory effects of PGA2 treatment indicates that PGA2 directly targets Ras, a molecule required downstream of Ras action, or a molecule involved in an entirely separate pathway required for cell cycle progression from G1 to S phase. This result is the opposite of that observed with TGF-β. In mink lung epithelial cells, the proliferation inhibitory action of TGF-β was completely overcome by injection of oncogenic Ras (30). These results were extended by demonstrating that NIH 3T3 cells transformed by oncogenic raf were also inhibited by PGA2 treatment. This observation indicates PGA2 apparently acts downstream of Ras and even downstream of Raf. When E2F-1 and DP-1 or adenoviral E1A protein was injected into PGA2-treated cells, however, efficient entry into S phase was observed. The results reported here indicate that the target(s) of PGA2 action functions between these two markers of proliferative induction, Ras/Raf and the activity of cellular molecules triggered by E1A or E2F-1/DP-1.

A number of treatments are known to block cell cycle progression in mid to late G1. In Balb/c 3T3 cells, treatment with sodium butyrate or a combination of the ion channel blockers amiloride and bumetanide efficiently block cell cycle progression in mid-G1 at a point which is prior to the requirement of Ras in late G1 (31). Interestingly, the effects of these inhibitors were not overcome by injection of either oncogenic Ras or E1A.

We interpret this observation to indicate that these two inhibitory mechanisms are independent events. The question of why the interaction of these two systems occurs and what the molecular target(s) of these inhibitors are remains to be elucidated.
itors block metabolic processes essential during preparation for cell cycle transit, processes such as the duplication of important cellular components. PGA2, on the other hand, apparently interferes with signaling or cell cycle control molecules which directly regulate transition between different phases of the cell cycle, leaving all preparatory metabolic requirements unaffected as indicated by the fact that E2F-1/DP-1 or E1A could overcome PGA2 inhibition.

A number of biochemical markers of proliferative signaling and cell cycle control were analyzed next. MAP kinase, whose activity is stimulated by the Ras-Raf-MEK pathway, was shown to be unaffected by PGA2 treatment, while the kinase activity of both cyclin D/CDK4 and cyclin E/CDK2 were completely inhibited, as was the hyperphosphorylation of pRb. Although it is p130, not pRb, which is primarily associated with E2F in G1 phase of mouse cells (37), there are several reasons to believe that pRb functions as one of the regulators of E2F in these cells and can serve as an indicator of the activity of the class of E2F regulating proteins. It has recently been shown that dihydrofolate reductase, an enzyme whose expression is regulated by E2F, is expressed at higher levels in pRb negative murine cells than normal cells (38). In addition, over expression of cyclin D in murine cells, which presumably induces hyperphosphorylation of pRb (6), can induce the activity of a reporter gene controlled by an E2F promoter (39). The facts that PGA2 blocks the hyperphosphorylation of pRb, and that E2F-1/DP-1 or E1A microinjection overcomes PGA2-induced growth arrest, are taken to indicate that PGA2 ultimately blocks the normal release of E2F-1/DP-1.

A possible explanation for the inhibition of G1 phase cyclin-CDK kinase activity by PGA2 is the induction of p21Waf1/Cip1/Sdi1 protein, since this protein has been shown to inhibit both cyclin D and CDK2 activity (33), and its binding to CDK2 prevents the activating phosphorylation by CDK activating kinase (40). In this study we have shown that PGA2-induced G1 arrest was not overridden by oncogenic Ras. On the other hand, although TGF-β also induces cyclin inhibitory proteins expression levels including p21Waf1/Cip1/Sdi1 (41), Ras can override TGF-β-induced G1 arrest (30). A possible interpretation of these observations is that interruption of mitogenic signaling at any site along the pathway, upstream of Ras in the case of TGF-β and downstream of Ras in the case of PGA2, may result in the up-regulation of CDK inhibitory protein(s). If so, p21Waf1/Cip1/Sdi1 induction might be a consequence rather than a cause of inhibition of the proliferative process. On the other hand, the possibility that PGA2 directly induces p21Waf1/Cip1/Sdi1 synthesis is supported by the fact that other genes are reported to be induced by PGA2 treatment, including gadd153, heat shock proteins, and hemoxylase (42–44).

We conclude that PGA2 is targeting downstream of most elements of the Ras-Raf pathway and elements prior to or at the site of the activation of most cyclins and associated kinases. Consequently, PGA2 apparently interrupts an activity which might be closely related to the point at which these two critical cell proliferation control mechanisms (proliferative signaling and cell cycle regulation systems) interact. Interestingly, however, microinjection of either E2F-1 or DP-1 alone could not efficiently override PGA2 cell cycle arrest while each was able to induce S phase efficiently in serum-starved, quiescent cells. This may indicate the possibility that PGA2 blocks the cell cycle not only by disrupting the connection between the two prolifera- tion control systems described above, but also by affecting the interaction of the cell cycle controlling system and the DNA synthesis process, where pRb, pRb-related proteins, and E2F/DP transcription factors are involved (9). Another possible explanation is that by suppressing the cyclin E-CDK2 activity, PGA2 can shut off the positive feedback loop of cyclin E, pRb, and E2F proposed by DeGregori et al. (45), which may result in the insufficient release of free E2F/DP transcription factor. On the other hand, since the E2F-1/DP-1 complex has more potent transactivation activity than each protein alone (7, 46), E2F-1/DP1 combined microinjection may readily be able to induce the molecule required for S-phase without this cyclin E-pRb-E2F amplifying loop.

In this study we have not tested the possible involvement of PGA2 in several other pathways with potential to modulate the activity of the cell proliferation controlling molecules discussed above. For instance, cyclic AMP is inhibitory in many cell types, and it has been suggested that PGA2 might function by elevating the intracellular concentration of cyclic AMP. Since anti-proliferative prostaglandins have been reported to arrest cell growth without cyclic AMP elevation (47), cyclic AMP may not be a mediator of PGA2 growth inhibition. Furthermore, recent studies suggest that cyclic AMP exerts its cell growth inhibitory effect by protein kinase A-dependent phosphorylation of either the regulatory domain or the kinase domain of Raf protein, resulting in either disruption of the association between Ras and Raf (48–50) or inhibition of the Raf kinase activity including v-Raf (51), respectively. Since MAP kinase activation requires Raf activity (48, 50, 52), and since PGA2 did not inhibit the serum-induced MAP kinase activation, neither of cyclic AMP-dependent Raf inactivation mechanisms appears to be involved in PGA2 growth arrest.

While it is unlikely that PGA2 functions through cyclic AMP, there are numerous signaling systems functioning within the cell which affect proliferation. Some of these, such as the stress kinase family, are likely to affect proliferative signaling (53). Others, such as small GTP-binding proteins of the Rho family, are likely to be required for proliferation in some cells (54). Finally, the cytokine signaling pathway might function either to enhance or interfere with proliferative signaling. The evidence above makes it clear that PGA2 affects a molecule closely related to the point of connection between the oncogene signaling pathway and the action of cyclins and related proteins. This might be accomplished by its ability to interact directly with a molecule whose action is closely related to the linkage between these two cell proliferation control systems, or it might be due to its ability to modify a separate signaling system which ultimately affects such a linkage molecule(s). Our data raise the possibility that p21Waf1/Cip1/Sdi1 might be one of such linkage molecules. In any case, it is likely that PGA2 will provide a valuable tool in unraveling the important mechanism by which the signal from proto-oncogenes alters the activity of cyclins and their associating proteins, thereby directly controlling passage through the cell cycle.

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