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Induction of *HSP70* Gene Expression by the Antiproliferative Prostaglandin PGA_2: A Growth-Dependent Response Mediated by Activation of Heat Shock Transcription Factor

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Prostaglandins (PG) of the A series are potent inhibitors of cell proliferation. Recently, it was shown that PGA_2-induced growth arrest was associated with the increased synthesis of stress proteins encoded by the *HSP70* gene family. In this study, we have examined the molecular basis for this increased HSP70 expression. Northern (RNA) blot analysis and nuclear run-on assays demonstrated that induction of high levels of *HSP70* mRNA results from an increase in the rate of transcription. High-level induction is specific to the HSP70 family of heat shock proteins and is rapid, reversible, dose dependent, and specific for PGs capable of growth-arresting HeLa cells. In addition, the response was found to be highly dependent on the growth state of the cells, as induction occurs in growing but not in confluent nongrowing cell populations. Induction is dependent on the activation of heat shock factor. Cycloheximide pretreatment, which inhibits the antiproliferative effects of PGA_2, prevents activation of the heat shock factor and induction of *HSP70* mRNA by PGA_2. These results support a role for HSP70 in mediating the antiproliferative effects of PGA_2.

The cyclopentenone prostaglandins (PG) PGA_1 and PGA_2 have been shown to be potent inhibitors of proliferation in a variety of cultured cells (2–4, 15, 37). These metabolites of PGE_1 and PGE_2, respectively, are characterized by the presence of an α,β-unsaturated carbonyl group in the cyclopentenone ring which is believed to be the active moiety in suppressing growth (3, 19, 24, 33). They have been found to inhibit the growth of B16 melanoma and Friend erythroleukemia virus-infected cells in vivo as well as in vitro, suggesting their utility as effective antineoplastic agents (15, 26, 39). They are actively taken up by cells via a specific carrier on the cell membrane and are transported to the nucleus, where they associate with nuclear proteins (30–32). They affect cell cycle progression in two ways. They act on cells at any stage of the cell cycle to cause a general decrease in the rate of progression, and they act specifically on cells in the G_1 phase to arrest progression (2, 35). Growth arrest has been associated with a decline in both c-*myc* and N-*myc* expression (22, 27). However, the exact mechanism by which PGs control proliferation is largely unknown.

The heat shock protein 70 (*HSP70*) gene family is part of a larger set of proteins whose syntheses are increased in response to a variety of chemical and biological stresses (25, 40). The expression of these genes follows the stress-induced activation of one or more heat shock transcription factors (HSF) which bind to a specific DNA sequence, the heat shock element (HSE), in the promoter regions of the *HSP* genes to increase their rates of transcription (36, 41, 42). There is also evidence to suggest that there are both positive and negative roles for HSPs in normal cell growth and differentiation. For example, certain HSPs have been shown to be induced in response to proliferative stimuli (12, 44). The transcriptional elements controlling this growth-related induction for the *HSP70* gene have been identified and are distinct from the HSE (29, 43). Other studies indicate that HSP70 expression also increases as cells enter a quiescent state (20, 21, 23).

Recently, two groups have reported that PGAs induce the synthesis of proteins of the HSP70 family (34, 38). This increase in protein synthesis is correlated with an inhibition of cellular proliferation, although a causal relationship between HSP70 expression and growth arrest has not been established, nor has the mechanism by which these PGs induce HSP70 expression been determined. This study was undertaken to examine the molecular basis for altered HSP70 expression following PGA_2 treatment. Using Northern (RNA) blot analysis and nuclear run-on assays, we demonstrate that PGA_2 induces high levels of *HSP70* mRNA which result from an increase in the rate of *HSP70* gene transcription. We provide evidence that this induction is dependent on protein synthesis and occurs through the interaction of the HSF with the HSE in the *HSP70* promoter. Importantly, the ability of PGA_2 to induce *HSP70* gene expression was found to be highly dependent on the growth state of cells occurring in proliferating but not in confluent cells.

MATERIALS AND METHODS

Cell culture conditions and treatments. HeLa S3 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories) supplemented with gentamicin (50 μg/ml) and 10% fetal bovine serum (HyClone Laboratories). PGs (Sigma Chemical Co.) were prepared as stock solutions in 95% ethanol and used at the indicated concentrations. Actinomycin D (1 μg/ml) and cycloheximide (8 μg/ml) were obtained from Sigma. Heat shock treatments were performed as previously described (11).

RNA isolation and analysis. RNA was isolated by lysis and

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disruption of cells in guanidine isothiocyanate followed by centrifugation through cesium chloride cushions (6). Northern blot hybridization and dot blot analyses were performed with total RNA as previously described (11). Hybridization signals were quantified by densitometric measurements of autoradiographs. To control for variation in either the amount of RNA in different samples or loading errors, all blots were hybridized with an oligonucleotide probe corresponding to 18S RNA. All densitometric values for HSP70 and expression were normalized to values for 18S RNA obtained on the same blot.

cDNA probes. A hamster *HSP70* cDNA probe was used to detect *HSP70* transcripts (13). cDNA probes for HSP27, HSP89α, and HSP89β were obtained from StressGen Biotec. Corp. The human β-actin cDNA was obtained from L. Kedes (Stanford University School of Medicine). cDNA probes were labeled with [^{32}P]dCTP, using the random primer labeling kit from Boehringer Mannheim. The 24-bp oligonucleotide (5′-ACGGTATCTGATCGTCTTCGAACC-3′) complementary to 18S RNA was synthesized with an ABI 391 DNA synthesizer (Applied Biosystems, Inc.) and labeled at the 3′ end with terminal deoxynucleotidyltransferase.

Nuclear run-on assays. Nuclei were isolated from cells by detergent lysis and stored at −80°C in storage buffer containing 40% glycerol (16). The transcription reaction and isolation of labeled RNA were performed according to the procedure of Celano et al. (5) except that the reaction was performed at 30°C for 30 min in the presence of 100 μCi of [α-^{32}P]UTP. For each sample, 10 μg of linearized plasmid DNA was bound to Genescreen Plus membranes according to the manufacturer's protocol. Duplicate filters were incubated with equivalent amounts (disintegrations per minute) of labeled RNA in 6× SSPE–0.5% sodium dodecyl sulfate (SDS)–100 μg of salmon sperm DNA per ml–50% formamide–5× Denhardt's solution for 48 h at 42°C. The filters were washed in 2× SSC–1% SDS at 42°C for 30 min and then in 1× SSC–1% SDS at 65°C for 30 min.

Assay for tritiated thymidine incorporation. DNA synthesis was determined by [*methyl*-^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material. Twelve hours after cells were plated in replicate wells (2.0×10^4 cells per well) of 24-well plates, PGA$_2$ (36 μM) and 4 μCi of [^{3}H]thymidine were added to each experimental well. Only the [^{3}H]thymidine was added to control plates. Cells were incubated for the indicated time periods, after which the reaction was stopped by the addition of 10% trichloroacetic acid. Cells were dissolved in 500 μl of 0.1 N NaOH and neutralized with 0.1 N HCl; 200 μl of sample was then counted by scintillation spectroscopy.

Transfections and CAT assays. The LSNwt HSP-chloramphenicol acetyltransferase (CAT) construct (29) was kindly provided by Richard I. Morimoto (Northwestern University). HeLa cells were transfected with 5 μg of CsCl-purified plasmid DNA, using the $CaPO_4$ precipitation procedure as previously described (18). Twenty-four hours after transfection, cells were treated with PGA$_2$ or were heat stressed. Cells were harvested at the indicated times and assayed for CAT activity as previously described (18).

Gel retardation assays. Gel retardation assays were performed on whole cell lysates as previously described (1) with a double-stranded oligonucleotide corresponding to the HSE consensus sequence (5′-GCCTCGAATCGCGAAGTTTCG-3′).

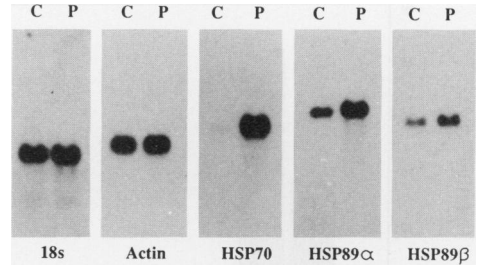

FIG. 1. Northern blot hybridization analysis of RNA from control and PGA$_2$-treated HeLa cells. Exponentially growing cells were treated for 10 h with 36 μM PGA$_2$ or vehicle (0.1% ethanol) prior to RNA extraction. Replicate samples (10 μg of total RNA) from control and PGA$_2$-treated cells were electrophoresed on a single gel and transferred to nylon membranes. Strips of the membranes containing a control (C) and a PGA$_2$-treated (P) lane were hybridized with radiolabeled cDNA probes specific for *HSP70*, *HSP89*α, *HSP89*β, β-actin, and 18S RNAs.

RESULTS

PGA$_2$-induced expression of *HSP70* mRNA in HeLa cells. Consistent with previous observations by others (35), we have found that treatment of HeLa cells with PGA$_2$ results in a marked inhibition of cell growth (7). Figure 1 shows the effect of PGA$_2$ on expression of various *HSP* mRNAs in HeLa cells. As can be seen, PGA$_2$ treatment resulted in a large increase in *HSP70* mRNA levels, but other *HSP* mRNAs were less affected. *HSP70* mRNA was induced 20-fold, while *HSP89*α and *HSP89*β mRNAs were induced only 3.3- and 1.7-fold, respectively. In other experiments, we found that *HSP27* mRNA was induced 1.9-fold by PGA$_2$ (data not shown). β-Actin levels were not affected by the PGA$_2$ treatment.

Figure 2 shows the dose-response relationship for induction of *HSP70* mRNA following treatment of HeLa cells with PGA$_2$ concentrations ranging from 3 to 72 μM. Consistent with previous reports on the induction of HSP70 protein synthesis by PGA$_2$ (34), significant mRNA induction was

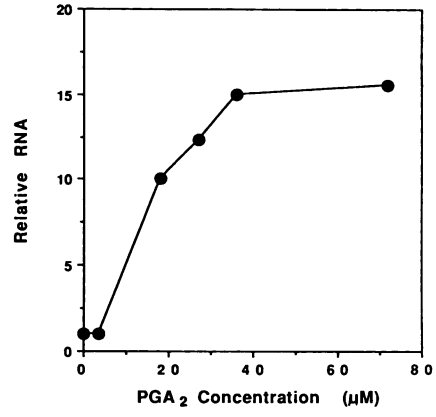

FIG. 2. Dose-response relationship for induction of HSP70 in HeLa cells by PGA$_2$. The relative abundance of RNA from PGA$_2$-treated samples was determined by RNA dot blot hybridization. Values are expressed as the ratio of hybridization signal (optical density) seen for RNA from treated cells and RNA from untreated exponentially growing cells isolated at the same time. All values were normalized to those obtained from hybridization to an 18S RNA probe to control for quantitation and loading errors.

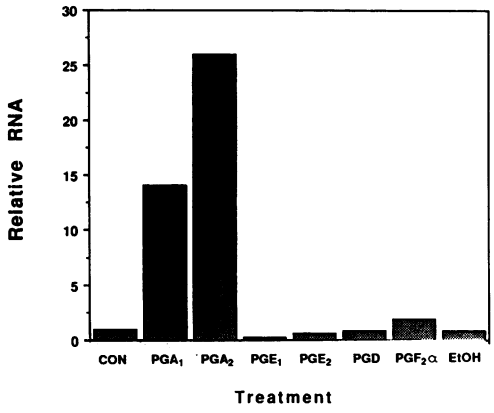

FIG. 3. Induction of HSP70 by different PGs. The relative abundance of RNA from PG-treated samples was determined as described in the legend to Fig. 2. The concentration of ethanol (ETOH) was 1% (vol/vol), the highest concentration used as diluent for the different PGs. CON, control.

seen with 18 μM PGA_2, with maximum expression obtained with 36 μM PGA_2.

A variety of other PGs were tested for their ability to induce *HSP70* transcripts (Fig. 3). Only PGA_1 and PGA_2, those PGs known to induce growth arrest, were effective in inducing HSP70 expression.

Kinetics of induction and its reversal. We next determined the kinetics of HSP70 induction following treatment of cells with PGA_2 (Fig. 4A). Relatively low levels of expression were observed in untreated, growing cells. Following PGA_2 treatment, induction was rapid, with clear increases in *HSP70* mRNA noted as early as 90 min following addition of the PG, although maximal expression required 8 h of treatment. The response was transient in nature, declining to about half maximum by 24 h. These results are consistent with those seen for HSP70 protein in which levels begin to rise at 3 h, reach a maximum at 10 to 12 h, and decline thereafter (34).

Earlier reports demonstrated that the growth-inhibitory effects of PGAs are reversible (33, 37, 38). Therefore, we examined the expression of *HSP70* mRNA following the removal of PGA_2. As seen in Fig. 4B, when PGA_2 was removed after 8 h of treatment, there was a rapid decrease in the expression of *HSP70* transcripts, much faster than the decline that occurs when the PG is left in cultures (Fig. 4A). The rapid induction of HSP70 expression following PGA_2 treatment and the rapid decline in *HSP70* mRNA levels following PGA_2 removal correlated well with the inhibitory effect of PGA_2 on tritiated thymidine incorporation (Fig. 4C). Thus, the expression of *HSP70* mRNA is tightly correlated with the inhibition of cell proliferation.

PGA_2-induced HSP70 expression is transcriptionally mediated and requires de novo protein synthesis. The induction of *HSP70* mRNA by PGA_2 was completely blocked by 1 μg of actinomycin D per ml, indicating that RNA synthesis was required for the effect (Fig. 5A). To determine whether de novo protein synthesis was necessary for the induction, cells were treated with PGA_2 in the presence of cycloheximide (8 μg/ml). The protein synthesis inhibitor completely blocked the induction of *HSP70* mRNA by PGA_2 (Fig. 5A).

Nuclear run-on experiments demonstrated that the induction was due to an increased rate of transcription of the *HSP70* gene (Fig. 5B). Consistent with steady-state mRNA levels, the rate of actin transcription did not vary significantly with PGA_2 treatment. No significant hybridization signal was detected for the pBR322 vector. Time course studies revealed that the maximum rate of transcription occurred at about 6 h and then declined (Fig. 5C). These findings are consistent with the steady-state mRNA levels, which were maximal about 8 h following treatment and declined at 24 h. Thus, it appears that the major control of HSP70 induction by PGA_2 occurs at the level of transcription.

In additional experiments, we examined the effect of PGA_2 treatment of cells on the activity of the human *HSP70* promoter, using the LSNwt reporter gene in which the *HSP70* promoter sequences from −188 to +150 were fused to the CAT reporter gene. This construct has been shown to contain the *cis* elements necessary for activation of *HSP70* gene expression by heat stress in HeLa cells (29). As shown in Table 1, in cells transiently transfected with LSNwt, *HSP70* promoter-driven CAT activity was increased threefold following treatment of the cells with PGA_2. This is about one-half the level of CAT activity seen following treatment of transfected cells with heat stress, which resulted in a six- to eightfold increase in promoter activity.

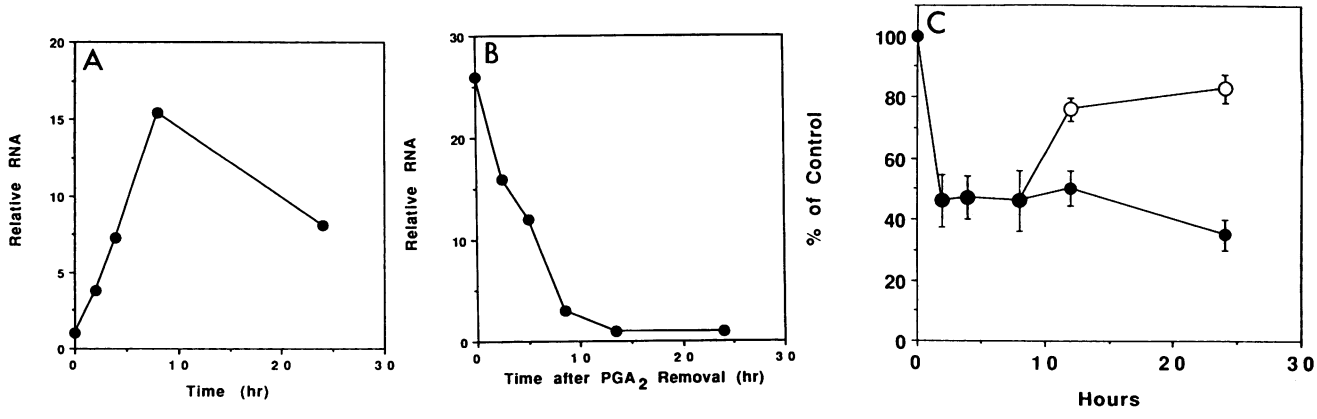

FIG. 4. Time course and reversibility of the PGA_2-induced expression of *HSP70* mRNA. (A) Exponentially growing cells were treated with 36 μM PGA_2 for the indicated times. (B) Cells were treated with 36 μM PGA_2 for 8 h, at which time the PG was removed (time 0). Relative RNA levels were determined at the various time points following the removal. (C) Effect of PGA_2 on DNA synthesis. Symbols: ●, cells continuously exposed to PGA_2; ○, cells from which PGA_2 was removed at 8 h.

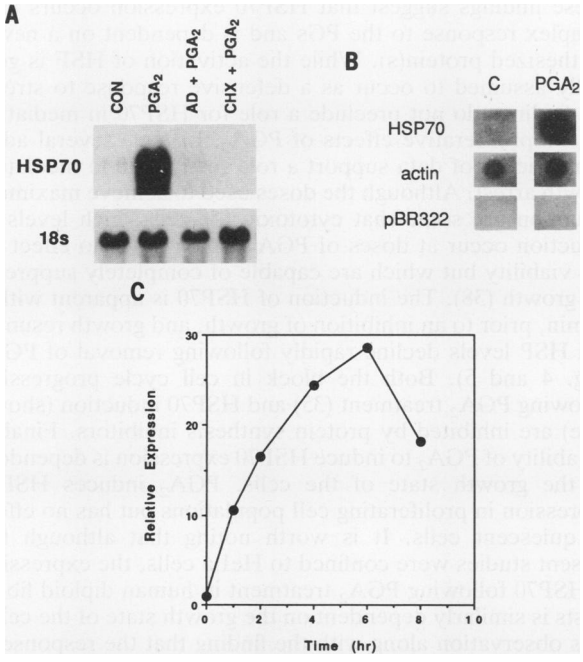

FIG. 5. Transcriptional regulation of PGA$_2$-induced expression of HSP70. (A) Effects of actinomycin D (AD; 1 μg/ml) and cycloheximide (CHX; 8 μg/ml) on PGA$_2$-induced *HSP70* mRNA expression determined by Northern blot hybridization analysis. Cells were treated with the inhibitors 30 min prior to addition of PGA$_2$ (36 μM). RNA was harvested after 8 h of treatment with the PG. CON, control. (B) In vitro transcription in isolated HeLa cell nuclei following PGA$_2$ treatment. Nuclei of untreated cells and cells treated with PGA$_2$ for 6 h were concurrently isolated. ^{32}P-labeled RNAs were hybridized to filter-bound DNAs of HSP70 and actin. C, control. (C) Quantitation of *HSP70* transcription rates in PGA$_2$-treated cells as measured by scanning densitometry.

PGA$_2$ does not induce HSP70 expression in confluent medium-depleted HeLa cells. To determine the influence of growth state on the induction of HSP70 by PGA$_2$, we determined the effect of PGA$_2$ on HSP70 expression in HeLa cells made quiescent by growing them to high density and maintaining them without refeeding for 3 days. We have previously shown that these conditions are sufficient to induce the expression of several growth arrest-specific genes in various cell types, including HeLa (7, 14). As shown in Fig. 6A, untreated confluent cultures expressed lower basal levels of *HSP70* mRNA than did exponentially growing cells. Importantly, in confluent cultures, PGA$_2$ treatment did not induce HSP70 expression, while in proliferating cultures, high levels of *HSP70* mRNA were expressed. In contrast, induction of *HSP70* mRNA in response to heat was not affected by the growth state (Fig. 6B). Results for β-actin mRNA expression obtained by reprobing the same blot showed that actin levels also declined as a function of quiescence. However, β-actin mRNA levels were similar in PGA$_2$-treated and control cells under both conditions.

TABLE 1. Transient expression of transfected LSNwt in HeLa cells following treatment with PGA$_2$ or heat stress

Expt	Relative CAT activity[a]		
	Control	PGA$_2$[b]	Heat stress[c]
1	0.8	2.5 (3.1)	6.6 (8.2)
2	0.5	1.3 (2.9)	2.6 (5.7)

[a] Values are actual percent conversions obtained in the CAT enzymatic assay following treatment of HeLa cells transiently transfected with LSNwt. Numbers in parentheses are fold increases in CAT activity seen in treated cells relative to untreated cells.

[b] At 24 h after transfection, cells were exposed to 36 μM PGA$_2$ for 8 h.

[c] At 24 h after transfection, cells were placed at 42°C for 1 h and then allowed to recover for 7 h at 37°C before harvesting for CAT activity.

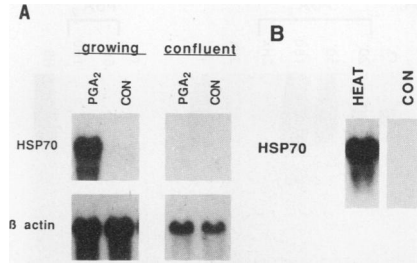

FIG. 6. Induction by PGA$_2$ of HSP70 expression in growing but not confluent HeLa cells. (A) Exponentially growing and confluent HeLa cells were treated with 36 μM PGA$_2$ for 8 h prior to extraction of RNA and Northern blot hybridization analysis. (B) Confluent cultures were heat stressed at 42°C for 90 prior to analysis for HSP70 expression. CON, control.

PGA$_2$ activates HSF. To determine whether PGA$_2$ induces HSP70 expression through the activation of HSF, we examined the levels of HSE-binding activity in whole cell extracts from control and PGA$_2$-treated cells, using the gel mobility shift assay. In addition, since the results shown in Fig. 6 indicated that HSP70 expression was induced by PGA$_2$ in growing but not in confluent cells, we compared the levels of HSE-binding activity with these two growth states. The HSE consensus sequence used was derived from the *Drosophila HSP70* gene and has been shown to bind to the heat shock-activated HSFs of a variety of mammalian species, including human and rodent (1, 8). As shown in Fig. 7, within 1 h of PGA$_2$ treatment, high levels of HSE-binding activity were present in extracts from growing cells. The level of binding activity was similar to that seen following exposure of cells to 42°C for 1 h and increased to much higher levels with 3 h of PGA$_2$ treatment. Consistent with the requirement for de novo protein synthesis for mRNA induction (Fig. 5A), cycloheximide prevented the activation of HSF by greater than 90%. Importantly, activation of HSF also did not occur in confluent cells treated with PGA$_2$ (which also did not express *HSP70* mRNA [Fig. 7C]), although it was present in confluent cells exposed to heat stress. The specificity of the HSE-binding activity is demonstrated in Fig. 7B. A 25-fold excess of cold HSE competed effectively for the binding to labeled HSE, whereas 50-fold excesses of three unrelated sequences corresponding to other known transcriptional factors did not. Thus, the binding observed in these experiments represents authentic HSE-HSF interactions.

DISCUSSION

Previous studies demonstrated that PGA$_2$-mediated growth arrest was associated with increased synthesis of HSP70 proteins (34, 38). This effect of PGA$_2$ was presumed to reflect an increase in *HSP70* mRNA synthesis, since actinomycin D blocked the increase in HSP70 protein expression, but no direct evidence was provided for transcriptional control. It was not determined whether the increase in HSP70 expression were causally related to the

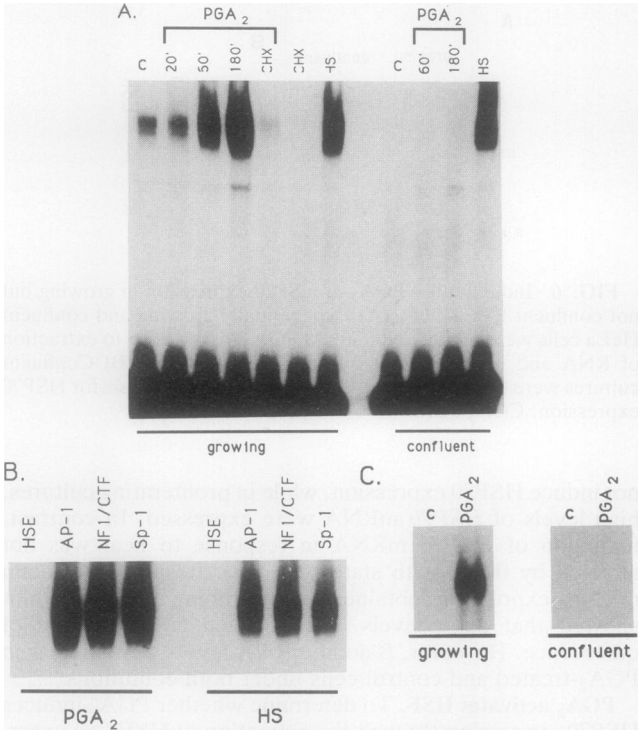

FIG. 7. Activation by PGA_2 treatment of HSF in growing but not confluent HeLa cells. (A) Whole cell extracts (1 μg of protein) prepared at various times following treatment with 36 μM PGA_2 were incubated with 1 ng of ^{32}P-HSE oligonucleotide. Cells were treated with PGA_2 for 20, 60, or 180 min or for 3 h with PGA_2 in the presence of cycloheximide (CHX), as indicated. C, control, untreated cells; CHX, control cells treated with cycloheximide; HS, cells subjected to heat stress (1 h at 42°C). (B) Specificity of the HSE-binding activity. Unlabeled oligonucleotides (25 ng [25-fold excess]) corresponding to HSE or binding sites of other transcriptional factors were added to the binding reaction from the lysates used in panel A. (C) *HSP70* mRNA expression in the same experiment. C, control.

state of growth arrest or occurred as a response to toxic effects of the compounds. To better understand the relationship between PGA_2-mediated growth arrest and HSP70 expression, we have examined the molecular basis for this increased expression. Consistent with the expression of HSP70 protein, the induction of *HSP70* mRNA following PGA_2 treatment is rapid, reversible, dose dependent, and specific for PGs capable of growth-arresting HeLa cells. We have demonstrated that induction is controlled at the level of transcription. Although the results presented here were limited to HeLa cells, it is important to note that both the inhibition of cellular proliferation and induction of HSP70 by PGA_2 are observed in all cell lines that we have studied, including a variety of transformed and untransformed human cell lines (Jurkat T lymphocytes, K562 erythroleukemia cells, normal keratinocytes, and lung diploid fibroblasts), Rat-1 fibroblasts, NIH 3T3 fibroblasts, and Chinese hamster ovary cells (not shown).

PGA_2 treatment leads to the activation of HSF, and this appears to be the primary mechanism responsible for induction of *HSP70* mRNA. However, this does not appear to be a direct effect of PGA_2 on the HSF, since unlike the general stress response elicited by heat and heavy metals, the activation of HSF by PGA_2 is prevented by cycloheximide. These findings suggest that HSP70 expression occurs as a complex response to the PGs and is dependent on a newly synthesized protein(s). While the activation of HSF is generally assumed to occur as a defensive response to stress, our findings do not preclude a role for HSP70 in mediating the antiproliferative effects of PGA_2. In fact, several additional pieces of data support a role for HSP70 in mediating growth arrest. Although the doses used to achieve maximum induction are somewhat cytotoxic for cells, high levels of induction occur at doses of PGA_2 which have no effect on cell viability but which are capable of completely suppressing growth (38). The induction of HSP70 is apparent within 60 min, prior to an inhibition of growth, and growth resumes and HSP levels decline rapidly following removal of PGA_2 (Fig. 4 and 5). Both the block in cell cycle progression following PGA_2 treatment (35) and HSP70 induction (shown here) are inhibited by protein synthesis inhibitors. Finally, the ability of PGA_2 to induce HSP70 expression is dependent on the growth state of the cells. PGA_2 induces HSP70 expression in proliferating cell populations but has no effect on quiescent cells. It is worth noting that although the present studies were confined to HeLa cells, the expression of HSP70 following PGA_2 treatment in human diploid fibroblasts is similarly dependent on the growth state of the cells. This observation along with the finding that the response is transient (even with chronic exposure to the PG) is consistent with the notion that HSP70 expression is involved in the entry of cells into a state of growth arrest, rather than occurring as a consequence of growth arrest. That is to say, that the process affected by PGA_2 exists only in dividing cells, and this process elicits the activation of the *HSP70* gene.

Although we cannot entirely rule out the possibility that the HSP70 expression by PGA_2 merely reflects a toxic effect of the compound, we think that this is unlikely for the following reasons. As already mentioned, induction occurs at nontoxic concentrations. The response to PGA_2 is rather selective for HSP70, whereas HSP70 and HSP90 are generally found to be coregulated in response to heat and other stresses (9, 10, 17). The selectivity of this response could reflect a differential threshold or sensitivity for transcriptional activation contributed by unique elements distinct from the HSE. If HSP70 expression were due simply to PGA_2 toxicity, then one would expect that it would also occur in confluent cultures as shown here for heat stress (Fig. 6 and 7). We have also found that the response of cells to other inducers of HSP70 such as cadmium chloride and arsenite is not dependent on the growth state (not shown). However, if HSP70 expression does result from toxicity attributed to this antiproliferative PG, then nongrowing cells are certainly less susceptible to such toxicity, and this has important implications with respect to the use of these PGs as antineoplastic agents. We tested the effect of 3-h PGA_2 treatment on freshly isolated minced lung tissue and saw no induction of HSP70, while cadmium chloride and sodium arsenite treatment induced high levels of *HSP70* mRNA expression. Presumably, the insensitivity of lung tissue to PGA_2 reflects the quiescent state of lung cells in vivo. Thus, HSP70 expression could serve as a useful marker for ascertaining the in vivo sensitivity of various neoplasms to these antiproliferative PGs.

PGA_2 is a naturally occurring metabolite of PGE_2 produced in vivo. However, the high concentrations necessary to achieve growth arrest make it unlikely that these PGs are involved in the regulation of proliferation in normal physiological states. Nonetheless, PGA_2-mediated growth arrest

provides an attractive model for unraveling the complex steps involved in cell cycle regulation and in particular for defining key factors involved in negative growth control. Many studies have suggested a role for HSP70 in regulating cell growth, but how they might function in controlling proliferation is entirely unknown. They have been both positively and negatively correlated with proliferation (reviewed in references 21 to 23, 25, 28, 43, and 44). Building on previous observations, our findings suggest that HSP70 is part of a novel regulatory pathway involved in the inhibition of cellular proliferation by PGA$_2$. Further delineation of this response could provide new insights into the function of HSP70 during normal cell growth as well as during stress.

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