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Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene

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ABSTRACT **Platelet-activating factor (PAF), a potent lipid mediator generated in cell injury and in the inflammatory and immune responses, promotes transcriptional activation of several primary response genes. *TIS10/PGS-2* is a primary response gene encoding the inducible form of prostaglandin synthase. The inductive effects of PAF and retinoic acid (RA), alone and in combination, were studied with the regulatory region of *TIS10/PGS-2* transfected into an exponentially growing glioblastoma–neuroblastoma NG108-15 hybrid in the human SH-SY5Y neuroblastoma or in the NIH 3T3 cell. RA alone exhibited only a small inductive effect. However, in the presence of RA (100 nM), a PAF-dependent (1–50 nM) synergistic activation of luciferase reporter constructs driven by regulatory regions of the *TIS10/PGS-2* gene was found. The hetrazepine BN-50730, an antagonist selective for intracellular PAF binding sites, inhibited PAF and RA induction of luciferase from the *TIS10/PGS-2* promoter. Thus, the intracellular PAF binding site is involved in *TIS10/PGS-2* expression. Induction is rapid, suggesting that the combination of PAF and RA activates a preexisting latent transcription factor(s). Deletion studies restrict the major PAF and RA cis-acting response element of the *TIS10/PGS-2* gene to a 70-nucleotide sequence as an intracellular inducer of *TIS10/PGS-2* expression. The synergistic effect of RA and PAF represents an unusual convergence of nuclear signaling pathways by which, through the modulation of preexisting transcription factors, specific gene expression can be upregulated. PAF-dependent induction of *TIS10/PGS-2* expression may play a role in cell injury, differentiation, inflammation, and immune responses.**

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a potent mediator of inflammatory and immune responses (1, 2), is rapidly produced in tissues in response to injury and various forms of stimulation. Intracellular and cell surface binding sites for PAF have been distinguished by structurally diverse PAF antagonists that show a preference for one or the other class of binding sites (3). However, unlike other lipid mediators (e.g., prostaglandins and lipoxygenase products), once formed, PAF is often retained in cells (4–6). This cellular retention is a prominent feature of stimulus-evoked PAF and may underlie intracellular functions (4, 7, 8). When PAF is added to cells (e.g., washed rabbit platelets), it is rapidly internalized (9). The identification of specific microsomal binding sites with the highest affinity for PAF thus far reported (2, 10) strongly suggests an intracellular role(s) for this lipid mediator. Thus far, the cloning of the cell surface PAF receptor, but not the intracellular PAF receptor, has been reported (9). Under some conditions, PAF is also released from cells (11) and may elicit intercellular functions (12).

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Intracellular PAF binding sites are linked to primary response gene expression. For example, PAF promotes transcriptional activation of both c-*fos*, c-*jun*, and *TIS1* (13–17). PAF-induced gene expression is inhibited by PAF antagonists (13, 15, 18), particularly the hetrazepine BN-50730, which is selective for the intracellular site (19). *In vivo* stimulation, such as chemically and electrically induced seizures, triggers both the generation of PAF (20) and the accumulation of c-*fos* mRNA (21–24) and *zif-268* mRNA (25, 26) in the brain. Intracerebroventricular injection of BN-50730 prior to stimulation partially inhibits the c-*fos* message accumulation and markedly inhibits *zif-268* expression (19), suggesting that PAF is an intracellular messenger that activates gene expression through a BN-50730 sensitive intracellular PAF receptor.

Retinoic acid (RA) participates in the morphogenesis, differentiation, and modulation of cell growth. RA modulates gene expression through nuclear receptors that are members of a superfamily of ligand-dependent transcription factors. Convergence of signal transduction pathways modulated by retinoids with pathways modulated by other ligands occurs through the formation of heterodimers that synergistically induce gene expression (27). It has been postulated that PAF-triggered gene expression may be affected by the convergence of multiple intracellular pathways (13). To elucidate the diversity of cellular responses stimulated by PAF, we have explored PAF and RA interactions in the induction of gene expression.

The inductive effects of PAF and RA, alone and in combination, were studied with the regulatory region of the gene for the inducible isozyme of prostaglandin synthase (PGS; prostaglandin-endoperoxide synthase; 5*Z*,8*Z*,11*Z*, 14*Z*-icosa-5,8,11,14-tetraenoate, hydrogen-donor:oxygen oxidoreductase, EC 1.14.99.1), *TIS10/PGS-2* transfected into exponentially growing cells using the calcium phosphate coprecipitation procedure. The *TIS10/PGS-2* primary response gene was identified as one of several phorbol ester (28) and serum (29) inducible genes in NIH 3T3 cells, and as a v-*src*-inducible gene in chicken embryo fibroblasts (30). The TIS10/PGS-2 enzyme and the constitutive isoform PGS-1 catalyze the cyclooxygenation and peroxidation of arachidonic acid, leading to synthesis of prostaglandins, prostacyclins, and thromboxanes. The present work describes a PAF-dependent activation of the *TIS10/PGS-2* promoter in the presence of RA and identifies a cis-acting region of the *TIS10/PGS-2* gene responsive to these signals.

MATERIALS AND METHODS

Materials. NG108-15, a mouse neuroblastoma–rat glioma hybrid cell line, was a gift from Marshall Nirenberg (National Institutes of Health, Bethesda, MD). The SH-SY5Y human

Abbreviations: PAF, platelet-activating factor; RA, retinoic acid; PDGF, platelet-derived growth factor.

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neuroblastoma cell line was a gift from Joan Biedler (Sloan–Kettering Memorial Institute, New York). *TIS10/PGS-2* promoters of 963 (L) or 371 (S) nucleotides fused to the luciferase reporter gene have been described (31). The pSV-β-galactosidase control vector driven by a simian virus 40 promoter was purchased from Promega.

PAF, RA, and phorbol 12-myristate 13-acetate were purchased from Sigma. Lyophilized PAF was dissolved in chloroform/methanol (9:1) at 100 mM and stored at −20°C. Serial dilutions of PAF were dissolved in chloroform/methanol in polypropylene tubes and dried under a stream of nitrogen gas. PAF dilutions were subsequently dissolved in 0.10% bovine serum albumin (fraction V; Sigma) in culture medium. Luciferin was obtained from Analytical Luminescence Laboratory (San Diego).

Cell Cultures. NG108-15 cells were maintained as monolayer cultures in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and HAT (100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine). SH-SY5Y neuroblastoma cells were maintained as monolayer cultures in Eagle's minimum essential medium supplemented with 10% heat-inactivated FBS. NIH 3T3 cells were maintained in DMEM with 10% newborn calf serum. Cells were seeded onto 75-cm^2 tissue culture flasks at a density of 2×10^5 cells in 20 ml of medium and were used at midlogarithmic phase for experiments.

Construction of the *TIS10* Promoter Deletion Series. Plasmid $TIS10_L$ was cut at the *Sma* I site at nucleotide −371 (nucleotide +1 is the start site of transcription). The cut plasmid was treated with *Bgl* I for lengths of time ranging from 1 to 6 min. The products were pooled into three separate tubes. The resulting pools were cut with *Hin*dIII, filled in with T4 polymerase, and religated. Six clones, which contained promoter inserts, were chosen with sizes roughly between 150 and 400 nucleotides and were subjected to double-stranded sequencing (Sequenase), using oligonucleotides within the region to determine the exact 5′ termination sites. The largest promoter deletions began at −300, the smallest began at −80. All the deletions joined the luciferase reporter at +70, the site of joining of the $TIS10_L$ luciferase construct from which the deletions were prepared.

Transient Transfection Assays. Cells were each plated at 5×10^5 cells per 60-mm tissue culture dish 24 hr prior to transfection. The cells were fed fresh medium (with 10% FBS) 2 hr prior to transfection.

Transfection of the CsCl purified plasmid (25 μg each of TIS10 luciferase constructs per transfection) was accomplished by incubating the cells in calcium phosphate dissolved in Hepes-buffered saline for 3–4 hr (32). The transfected cells were then rinsed with 10% (vol/vol) glycerol in Hepes buffer for 60 sec and washed with phosphate-buffered saline (PBS), and then fresh medium containing 10% FBS was added. Eight hours later, transfected cells received new medium containing 0.5% FBS in addition to PAF, RA, or BN-50730. To check for variations in transfection efficiency, a β-galactosidase reporter plasmid driven by a simian virus 40 promoter was cotransfected in each experiment. The cells were harvested 36 hr after transfection and washed twice with PBS before resuspending in lysis buffer.

Luciferase Assays. The cell pellet was resuspended in 500 μl of 2× luciferase assay buffer [Analytical Luminescence Laboratory (ALL) buffer, 0.2 M K_2PO_4/2 mM dithiothreitol/30 mM $MgSO_4$/10 mM ATP]. Cells were lysed by freeze–thawing. Cellular debris were pelleted by centrifugation in a Beckman centrifuge for 15 min at 1200 × *g*. The equivalent of 20 units of β-galactosidase, 20–30 μl of the supernatant after normalization of β-galactosidase, was used in each assay and was mixed with 70–80 μl of 2× ALL buffer. The reaction was initiated by the injection of 100 μl of 1 mM luciferin and the relative light units were determined by using an ALL luminometer recording over a 20-sec interval. The luciferase assays were performed on duplicate or triplicate plates and normalized for protein content with the Bio-Rad protein assay kit.

β-Galactosidase Assay. The level of β-galactosidase induction was measured according to the manufacturer's instructions. The standard assay was performed by adding 10 μl of cell extract into 300 μl of phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, 45 mM 2-mercaptoethanol, and 10 mM *o*-nitrophenyl β-D-galactopyranoside and incubating at 37°C for 30–45 min. The reaction was terminated by addition of 500 μl of 1 M Na_2CO_3, and the absorbance was read with a spectrophotometer at 420 nm. β-Galactosidase activity was determined by plotting on a standard curve.

Statistical Analysis. The effects of RA, alone and in combination with PAF and BN-50730, on reporter gene expression by the two plasmids were assessed by a two-way ANOVA in which the treatment group (untreated, RA, RA + PAF, or RA + PAF + BN) and plasmid ($TIS10_S$ or $TIS10_L$) were the main effects. After the ANOVA was found significant, differences between the level of reporter gene expression in each treatment group were tested by *t* tests of differences between least-square means (33).

RESULTS

RA induced the expression of the $TIS10_L$ (963 bp) and $TIS10_S$ (371 bp) of *TIS10/PGS-2* promoter luciferase reporter constructs transfected into neuroblastoma cells (Fig. 1). Induction by RA was maximal at a concentration of 100 nM and was less at higher concentrations. Both the $TIS10_S$ and $TIS10_L$ promoters were responsive to RA. Further studies were carried out using the NG108-15 cells because they were more responsive than the SH-SY5Y cells.

Using the calcium phosphate coprecipitation transfection procedure, PAF alone could not induce the *TIS10/PGS-2* luciferase chimeric gene after transfection into exponentially growing NG108-15 cells (Fig. 2). However, PAF and RA

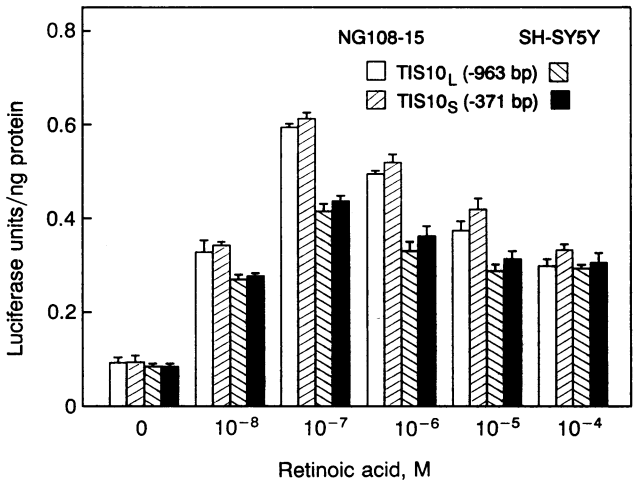

FIG. 1. RA induction of two different lengths of the *TIS10/PGS-2* promoter fused to the luciferase gene transfected in NG108-15 or SH-SY5Y neuroblastoma cells. NG108-15 and SH-SY5Y cells were transfected with $TIS10_L$, a plasmid in which the first 963 nucleotides of the promoter were fused to the luciferase reporter, or $TIS10_S$, a plasmid in which the first 371 nucleotides of the promoter were fused to the luciferase reporter. Luciferase activity in the transfected cells was measured as a function of concentration of RA during 12 hr of incubation. Values are means of triplicate plates in two independent experiments. Error bars indicate SD. Details of transfection, harvesting, and luciferase assays are described in *Materials and Methods*.

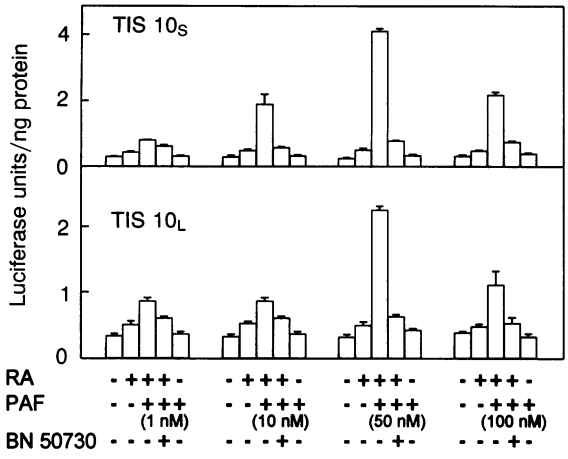

FIG. 2. PAF plus RA activate expression of *TIS10/PGS-2* promoter fused to the luciferase gene transfected in neuroblastoma cells. As described in *Materials and Methods*, NG108-15 cells were transfected with either $TIS10_L$ or $TIS10_S$ luciferase reporter constructs. The transfected cells were incubated with RA (100 nM) for 12 hr. After another 12-hr incubation with various concentrations (1–100 nM) of PAF, luciferase activity was determined. One hour before the addition of PAF, BN-50730 (100 nM) was added. Controls were mock transfected. Values are means of two independent experiments, with each having triplicate plates. All the RA plus PAF conditions shown are significantly different from the other conditions at the level of $P = 0.0001$ (t test of least-square means; ANOVA).

together elicited a synergistic induction of the *TIS10/PGS-2* promoter. RA (100 nM) was added to the cultures 12 hr before PAF in this experiment, and cells were harvested for luciferase analysis 12 hr after cells had been exposed to both inducers. Under these conditions, synergistic induction was PAF dependent, increasing between 1 and 50 nM PAF. At 100 nM, the effect of PAF decreased. A substantially greater luciferase induction was observed in cells transfected with $TIS10_S$ plasmid, in which the first 371 nucleotides of the *TIS10/PGS-2* promoter are fused to the reporter gene. BN-50730, an intracellular PAF binding site antagonist (19), inhibited the synergistic action of PAF plus RA seen with both constructs (S and L) at all the PAF concentrations studied.

To determine how rapidly the *TIS10/PGS-2* promoter would respond to PAF and RA in NG108-15 cells, cultures transfected with the $TIS10_S$ plasmid were preincubated with RA and PAF, added either together or sequentially, for various periods of time. Fig. 3*A* shows that, at the lowest effective PAF concentration studied (1 nM), there is a synergistic activation of the gene construct during 15 or 45 min of incubation in the presence of RA. After 12 hr of incubation under these conditions, a greater effect was observed. PAF (50 nM) plus RA activates the expression of either construct ($TIS10_S$ or $TIS10_L$) during 45 min of incubation. Once again, BN-50730 inhibited the synergistic induction of the *TIS10/PGS-2* promoter by the combination of RA and PAF (Fig. 3*B*). When either PAF or RA was added during the initial 45-min incubation, followed by a subsequent 45-min incubation with the other coactivator compound, the expression of both constructs was enhanced (Fig. 3*C*). It is noteworthy that $TIS10_L$ was less sensitive than $TIS10_S$ to PAF plus RA during this short time of incubation as compared with those depicted in Fig. 2. The rapid synergistic effect of RA plus PAF suggests that TIS10/PGS-2 induction by these ligands may not require protein synthesis and that the intracellular site of action may be a preexisting protein(s).

To analyze the specificity and potency of the induction of the TIS10/PGS-2/luciferase construct by RA plus PAF, we compared the induction by these ligands with that of platelet-

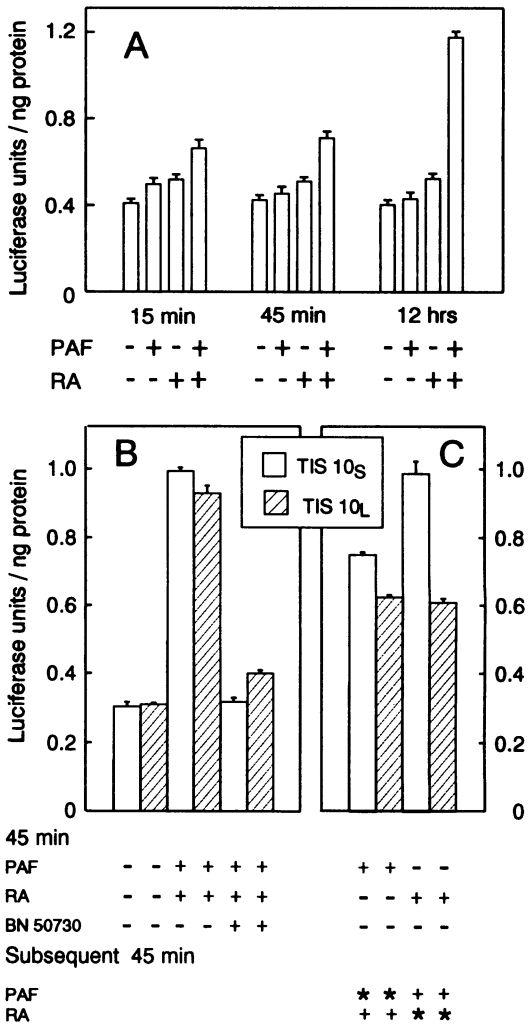

FIG. 3. Effect of incubation time and of simultaneous or subsequent addition of PAF and RA on *TIS10/PGS-2* luciferase construct. (*A*) NG108-15 cells were transfected with $TIS10_S$ luciferase reporter construct. Cells were harvested after 15- or 45-min or 12-hr incubation with PAF (1 nM), RA (100 nM), or PAF (1 nM) plus RA (100 nM). Then luciferase assays were performed. (*B*) NG108-15 cells were transfected with either $TIS10_S$ or $TIS10_L$ luciferase constructs as described. Transfected cells were then incubated with RA (100 nM) and PAF (50 nM). BN-50730 (100 nM) was added 1 hr before PAF and RA. (*C*) After transfection of both constructs, cells were incubated for 90 min. At time 0 either PAF (50 nM) or RA (100 nM) was added. After incubation for 45 min, RA (100 nM) or PAF (50 nM) was added to the plates that initially received either PAF or RA, respectively. Asterisks indicate continuous presence of the added ligand from time 0. Averages (±SD) from two experiments with three plates each are shown.

derived growth factor (PDGF), using NIH 3T3 cells (Fig. 4). The TIS10/luciferase constructs have previously been characterized in these cells (31). Induction by PDGF and the PAF and RA combination were approximately equivalent (Fig. 4). Moreover, BN-50730 did not block PDGF induction, but it did attenuate PDGF induction, suggesting that the RA plus PAF-mediated pathway is, at least in some step(s), distinct from the tyrosine kinase-mediated pathway of *TIS10/PGS-2* induction.

To map the RA plus PAF activating region, a 5′ deletion series of the *TIS10/PGS-2* promoter/luciferase reporter constructs were transfected into NG108-15 cells and challenged with a 12-hr preincubation with RA (100 nM) followed by a 12-hr exposure to PAF (50 nM). In this experiment, $TIS10_S$-driven luciferase activity stimulation was increased 31-fold

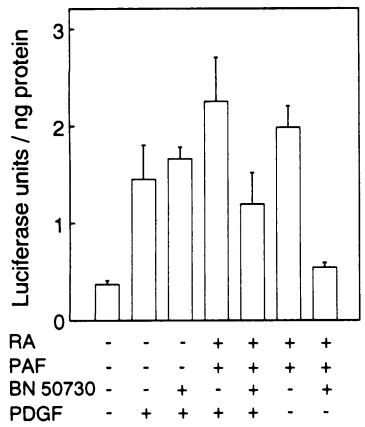

FIG. 4. Comparison between the effect of PAF plus RA with the effect of PDGF on the *TIS10/PGS-2* gene. NIH 3T3 cells were transfected with plasmid TIS10S by the calcium phosphate precipitation method. Four hours after transfection, the plates were washed with PBS and refed with fresh medium containing 10% bovine calf serum. Eight hours later, the transfected cells received new medium containing 0.5% bovine calf serum with no other additions (control), with PDGF (10 ng/ml), or with RA (100 nM) and PAF (50 nM). Some cells received 100 nM BN-50730 1 hr before addition of PDGF or RA and PAF. After 12 hr of incubation at 37°C, the cells were harvested and luciferase activity was measured. Data are averages (±SD) from two experiments with three plates each.

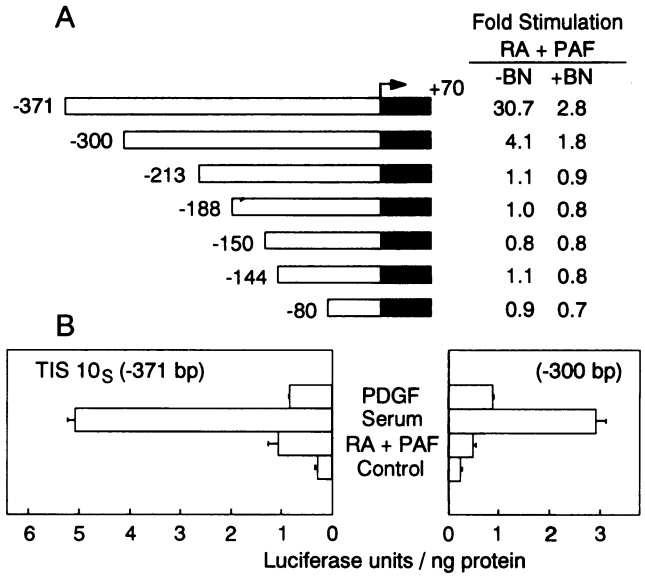

FIG. 5. Analysis of *TIS10/PGS-2* promoter deletion constructs and identification of a PAF-activating region(s). (*A*) *TIS10/PGS-2* promoter deletion–luciferase constructs were transfected into NG108-15 cells by the calcium phosphate coprecipitation method as described in *Materials and Methods*. Open box, *TIS10/PGS-2* promoter regions; solid box, luciferase reporter gene. Indicated below are the extent of the promoter deletion sequences of *TIS10/PGS-2* used in the transfection study. Transfected cells were treated with RA (100 nM) and PAF (50 nM) for 24 hr before assaying for luciferase activity in the cell lysates. BN-50730 (100 nM) was added 1 hr before the addition of PAF. The luciferase assay was performed with 20–30 μl of the crude cell extracts (20 units of β-galactosidase) as described. Values are means of triplicate plates in two independent experiments. (*B*) NIH 3T3 cells transfected with either TIS10S (−371 bp) or TIS10L (−300 bp). Incubation conditions were RA (100 nM) plus PAF (50 nM), serum (20%), and PDGF (10 ng/ml) for 6 hr. Controls were incubated with medium only. Treated cells were washed, harvested, and assayed for luciferase activity.

(Fig. 5). This activation was, once again, completely inhibited when BN-50730 (100 nM) was added to the cultures 1 hr prior to the addition of PAF. Deletion of the sequences between −371 and −300 of the *TIS10/PGS-2* promoter reduced the magnitude of induction to 4.1-fold, suggesting that a major PAF plus RA response site lies in this region. Further deletions completely eliminated the PAF plus RA induction response. Fig. 5*B* shows that the construct −300 bp showed lower activation by RA and PAF as compared with −371, whereas PDGF activated both constructs in a similar way. Serum, used as a positive control, showed the highest activation of these constructs.

DISCUSSION

The data presented here demonstrated a PAF-dependent activation of gene expression in the presence of RA, when added together or sequentially, in either order, to NG108-15 neuroblastoma cells or to NIH 3T3 fibroblasts transfected by the calcium phosphate coprecipitation procedure with constructs containing the promoter of TIS10/PGS-2. Interactions between RA and PAF on gene expression have not previously been demonstrated. The synergism is (*i*) dependent on PAF concentration; (*ii*) blocked by BN-50730, a potent antagonist of intracellular PAF binding sites (19); and (*iii*) detectable after only short incubation times following exposure to PAF and RA.

Microsomal fractions from rat cerebral cortex, purified in such a manner that they have limited plasma membrane contamination, contain specific binding sites displaying the highest affinity for PAF thus far reported. Specific PAF binding to microsomal fractions was 3-fold higher than values obtained for synaptosomal membrane binding sites (3). One possible role for intracellular PAF binding sites might be to couple a plasma membrane-activated signal with gene expression, using PAF as the intracellular second messenger (3, 13, 34, 35). In this regard, PAF mediates c-*fos*, c-*jun*, and collagenase type I gene expression in corneal epithelium (18). The specific PAF binding site in microsomal fractions is inhibited by increasing Ca^{2+} concentration (3). Thus, it is possible that the relatively high concentration of Ca^{2+} used in the present study for transfection may have affected that binding site. However, BN-50730, an antagonist selective for that site, inhibits the synergy of RA and PAF, indicating that even when PAF alone cannot exert induction by itself, under the present experimental conditions, RA and PAF together unmasked such a site or sites.

Induction of the TIS10S-driven luciferase activity by serum in NIH 3T3 cells is detectable at 45 min to 1 hr and peaks at 5–10 hr. Mitogen induction of *TIS10/PGS-2* transcription is not dependent on *de novo* protein synthesis, but it occurs as the result of activation of preexisting transcription factors (B.S.F. and H.R.H., unpublished data). These data suggest that the synergistic activation of *TIS10/PGS-2* by RA and PAF may not require the intervening step of protein synthesis but may result from the activation of a latent transcription factor(s) by the two ligands. Although many scenarios are possible, PAF and RA may bind to distinct or common proteins and thereby activate latent DNA binding and/or transcriptional activation proteins required for activation of the *TIS10/PGS-2* promoter. Heterodimer formation between thyroid hormone receptors, retinoid receptors, peroxisome proliferator-activated receptors, and vitamin D receptors has been demonstrated with the retinoid X receptor (27, 36–40). We speculate that a similar type of ligand-activated transcription factor may be a target for PAF and RA that subsequently may give rise to heterodimer or other complex protein conformations.

The PAF antagonist BN-50730 used in this study is able to inhibit electroconvulsive shock-triggered *zif-268/TIS8* gene expression in the hippocampus (19). Electroconvulsive shock also triggers a persistent enhancement of *TIS10/PGS-2* mes-

sage accumulation in hippocampus and cerebral cortex (ref. 41; and G. Allan and N.G.B., unpublished data). BN-50730 injected intracerebroventricularly inhibits accumulation of the *TIS10/PGS-2* message in the cerebrum after a cryogenic injury (V. L. Marcheselli and N.G.B., unpublished data). Since BN-50730 inhibits both *in vivo* induction of these primary response genes and the PAF plus RA-dependent induction of the *TIS10/PGS-2* gene in cultured NG108-15 and NIH 3T3 cells, it seems likely that the PAF-mediated event of these two induction paradigms may involve a common component. It is possible that in the *in vivo* experiments, endogenous RA or RA-like molecules may be either constitutively present or formed upon stimulation.

Induction of the $TIS10_S$ promoter construct by RA plus PAF is greater than that observed for the $TIS10_L$ construct. The shorter construct is also more inducible by phorbol 12-myristate 13-acetate or serum in Swiss 3T3 cells (38). These data suggest that there may be some element present in the distal portion (−963 to −371 bp) of the *TIS10/PGS-2* promoter that is responsible for general repression of this gene. The promoter deletion analysis presented in Fig. 5 suggests that a major cis-acting mediator of the RA plus PAF response lies between −371 and −300 nucleotides 5′ from the start site of transcription of the *TIS10/PGS-2* gene. This region does not contain any nucleotide sequence resembling RA receptor consensus binding sequences. Mutational studies of this region and electrophoretic mobility-shift analyses using nuclear extracts from induced cells will be required to identify both the critical nucleotides necessary for the cis-acting induction response to RA plus PAF and the transcription factors required for this induction.

These studies highlight an unusual convergence of signaling pathways to activate gene expression. The roles of RA and PAF in cell physiology and pathology are commonly thought to be widely different. PAF is rapidly formed in response to cell injury and is thought to play a role in inflammation and immune responses. RA, on the other hand, plays a role in differentiation. Induction of gene expression in response to extracellular ligands to mediate cellular physiology occupies a middle ground between these acute cellular defensive responses and long-term alterations in cell function. Our data suggest that components of both cellular responses may be involved in some aspects of primary response gene expression. This cross-coupling pathway to transcriptional activation is particularly interesting in the case of *TIS10/PGS-2*, because this gene encodes the inducible form of an enzyme that leads to the synthesis of prostaglandins, prostacyclin, and thromboxanes, another group of active mediators of both the differentiation and the inflammatory response. The PAF-dependent gene modulatory up-regulation of prostaglandin synthesis may represent a feedback system of these cellular responses.

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