Platelet-activating factor (PAF) is a phospholipid mediator with various physiological functions, including cellular growth and transformation. PAF exerts biological activities through G-protein-coupled receptors. In normal rat fibroblasts overexpressing a cloned PAF receptor, PAF induced immediate early oncogene expression and mitogenic responses. On the other hand, PAF strongly inhibited the epidermal growth factor-induced mitogenic growth response, growth acceleration, and anchorage-independent cell growth in a soft agar. Furthermore, PAF suppressed v-src- or v-ras-induced oncogenic morphological changes and anchorage-independent growth. Our observations suggest that PAF is a unique growth regulator with apparently diverse functions. Dual actions of PAF may relate to the point of action in the cell cycle; PAF stimulates the mitogenic response in G0-arrested cells in a pertussis toxin-sensitive manner, while it inhibits the G1 to S transition through a pertussis toxin-resistant manner.

Since discovery of its biological activities and identification of its chemical structure as a phospholipid (1, 2), platelet-activating factor (PAF)1 was found to play an important role in inflammatory and allergic responses (3), synaptic transmission (4–6), cardiovascular systems (7), and cellular growth and transformation (8–10). Cloning of the PAF receptor (PAFR) (11–15) revealed the rhodopsin-type structure, diverse second messenger systems induced following PAFR stimulation (16) and the involvement of PAF in transcriptional activations (17). PAFR is expressed in various organs and tissues (18), and the transcription is regulated by two distinct promoters in humans (19, 20). Using the cloned PAFR and its mutants expressed in Chinese hamster ovary cells, we showed that activation of PAFR leads to inhibition of adenylate cyclase, elevation of intracellular calcium concentration, arachidonic acid liberation, and mitogen-activated protein kinase activation. These intracellular effectors differed in their sensitivities to pertussis toxin (PTX) treatment (16, 21).

Activation of G protein-linked receptors stimulates cell growth, as do the growth factor receptors with intrinsic tyrosine kinase activities. A thrombin receptor induces mitogenic response in various types of cells (22). Lyso phosphatidic acid is considered to be the most potent growth factor in serum (23), and its receptors have been cloned (24, 25).

PAF activates mitogen-activated protein kinase and protein kinase C in Chinese hamster ovary cells expressing PAFR (16, 26), as well as in blood cells (27, 28). Transgenic mice overexpressing PAFR develop skin tumors (29), an event further supporting its role in control of cell growth.

In the present work, we used NRK cells to examine the effect of PAF on cellular growth. NRK cells are immortalized fibroblasts derived from the kidney of a normal rat. These cells are regarded as a pertinent model of growth and oncogenic transformation as their growth is tightly regulated like that of normal cells (30). They are contact-inhibited and do not grow in the absence of serum. They do not grow in soft agar either in an anchorage-independent manner. However, they can be reversibly transformed by various growth factors including epidermal growth factor (EGF), and irreversibly transformed by a variety of oncogenes such as v-K-ras and v-src (31). To delineate functions of PAF in cellular growth and transformation, we established NRK cells overexpressing PAFR and examined their response to PAF with respect to growth and transformation. We obtained evidence that this lipid mediator modulates cell growth in a dual manner. PAF stimulated the growth of G0-arrested cells in a pertussis toxin-sensitive pathway, while it inhibited the G1 to S transition and the accelerated cell growth of transformed cells through a pertussis toxin-insensitive pathway.

MATERIALS AND METHODS

Cell Culture and the Establishment of PAFR Stable Transformants—NRK cells (clone 49F, American Type Culture Collection) were maintained in 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum (FCS). PAFR expression plasmid was constructed by inserting guinea pig PAFR cDNA into pCXB2 expression vector (32), which contains cytomegalovirus enhancer, β-actin promoter, and β-globin polyadenylation signal for cDNA expression and neomycin resistant gene for selection (32). NRK cells were transfected with pCXB2-PAFR, using a LipofectAMINE reagent (Life Technologies, Inc.). Independent colonies were isolated using iron cylinders after selection by 1 mg/ml Geneticin (Life Technologies, Inc.) for 2 weeks, and 60 clones were tested for PAF receptor expression by [3H]WEB 2086 (DuPont) binding, as described (15, 16). Briefly, 1 × 105 cells were seeded in a 6-well culture plate, and incubated with 10 nM [3H]WEB 2086 (521.7 GBq/mmol) at 25 °C for 60 min. Cells were washed 3 times with 5 ml of phosphate-buffered saline, lysed with 1 ml of 10% Triton X-100, and radioactivity was counted. For the measurement of the nonspecific binding, 1 μM WEB 2086 was added. Clones
with high PAFR expression designated as NP3 and NP5 were used. PAF (C16) and methylcarbamyl-PAF (C-PAF) (C16) were purchased from Cayman Chemical, and EGF (mouse) was from Takara (Japan). WEB 2086 and BN50730 were generous gifts from Boehringer Ingelheim (Germany) and Dr. Nicholas Bazan (Louisiana State University), respectively. For the generation of oncogene-transformants, cells were transfected with a retroviral vector carrying v-K-ras or v-src, as described (33), and transformed foci were isolated.

**Northern and Western Blottings—**For Northern blot analysis, poly(A)\(^+\) RNA (1 \(\mu\)g) prepared with a QuickPrep Micro mRNA purification kit (Pharmacia Biotech) was separated on 1% agarose gel containing 3% formaldehyde, transferred to a nylon membrane (Paul) by capillary blotting followed by detection with \([^{32}\text{P}]\)dCTP-labeled probes, prepared with a Megaprime kit (Amersham). The probes used were human c-fos cDNA (Japan Cancer Research Bank, Japan), rat c-myc and rat cyclin D1 cDNA (gifts from Dr. Y. Terada, Okayama Cell Switching Project, Kyoto, Japan), and human glyceraldehyde-3-phosphate dehydrogenase cDNA (CLONTECH). For Western blot analyses, total cell lysate (50 \(\mu\)g) prepared in Tris-buffered saline containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 \(\mu\)g/ml leupeptin, and 0.7 \(\mu\)g/ml pepstatin-A was separated on 15% SDS-polyacrylamide electrophoresis gel, then transferred to a Immobilon polyvinylidene fluoride membrane (Millipore) by electroblotting. The blot was probed with a polyclonal anti-cyclin D1 (PRAD1) antibody (Santa Cruz), using an ECL system (Amersham), and according to the manufacturer’s protocol.

**Mitogenic Response and Cell Growth Assay—**Rapidly growing cells were trypsinized and seeded in a 96-well microplate (5,000 cells/well) in Dulbecco's modified Eagle's medium plus 0.5% FCS. After a 48-h culture, reagents for examinations were added. After 10 h, \([^{3}\text{H}]\)thymidine (0.5 \(\mu\)Ci/well, 20 Ci/mmol, DuPont) was added to each well and the preparations were incubated for an additional 8 h. Finally, cells were collected onto a 96-well format filter (Unifilter 96 GF/C, Packard) and washed with distilled water with a FilterMate Harvester and radioactivities were counted with a TopCount scintillation counter (Packard).

For growth curve analysis, rapidly growing cells were seeded in a 96-well microplate (500 cells/well) in Dulbecco’s modified Eagle’s medium plus 5% FCS. After the indicated days of culture, the medium was replaced with 150 \(\mu\)l of the medium without phenol red, supplemented with XTT (sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) and PMS (N-methyl dibenzopyrazine methyl sulfate) reagents (Cell Proliferation Kit II, Boehringer Mannheim). After a 3-h incubation at 37 °C, absorbance at 415 nm was measured using a microplate reader (Bio-Rad, model 3550). The absorbances obtained according to the manufacturer’s protocol proved to be proportional to the cell number, as determined in our preliminary control experiments (data not shown). For coating of plates used in the anchorage-independent growth assay, 50 \(\mu\)l of 5 mg/ml...
poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma) in 95% ethanol was placed on a 96-well plate, dried overnight at 37 °C, and used as described (34). For soft agar assay, 1 × 10³ cells were plated in 3 ml of growth media containing 0.33% Noble agar overlaid on 2 ml of pre-fixed bottom agar containing 0.5% agar in a 6-cm dish. Cell cycle analysis using a flow cytometry was performed using a fluorescence-activated cell analyzer (FACScan, Becton Dickinson), as described (35).

RESULTS

Establishment of PAFR-overexpressing Cells and Their Immediate Early Responses—NRK-49F cells were transfected with pCXN2-PAFR, and two clones with high PAFR expression, designated NP3 and NP5, were obtained, as described under “Materials and Methods.” These two clones possess 5.4 × 10⁶ and 4.3 × 10⁶ [3H]WEB 2086-binding sites per cell, respectively (the wild type NRK cells have less than 2 × 10³ sites). These numbers are higher than that (2.8 × 10³/cell) reported for rat peritoneal polymorphonuclear leukocytes (36). The growth rate and morphology of both NP3 and NP5 cells were not distinguishable from that of the wild type cells. Both clones showed a contact inhibition comparable to the wild type when they reached confluency in culture dishes, and the final cell densities of both clones did not differ significantly from that of the wild type.

Both clones, but not wild type NRK cells, responded to 1 nM PAF by evidence of elevated intracellular calcium concentrations, as examined using Fura-2 (data not shown). As shown in Fig. 1, c-fos and c-myc mRNAs were induced when NP3 and NP5 cells, preincubated with a low concentration of FCS (0.5%) for 48 h, were stimulated either by PAF or a synthetic PAF agonist, C-PAF. PAF and C-PAF similarly induced the activation of mitogen-activated protein kinase, as demonstrated by the mobility shift of erk1 and erk2 kinases in NP3 and NP5 cells, but not in wild type NRK cells (data not shown). These immediate responses are evidence of the expression of a functional PAFR.

Stimulation and Inhibition of Mitogenic Response of NRK Cells—Mitogenic responses of PAFR-expressing NP3 and NP5 cells were then examined. The cells were growth arrested at G0 by incubation with 0.5% FCS for 48 h, then various concentrations of PAF were applied. After a 10-h incubation, [3H]thymidine was added and cells were harvested after a further 8-h incubation. As shown in Fig. 2A, low concentrations (up to 100 nM) of PAF induced a significant mitogenic response. C-PAF, a synthetic PAF analogue and a metabolically stable PAFR agonist, did not induce a growth response, at any concentration used (Fig. 2B). Because EGF induces mitogenic responses of NRK cells (30), PAF or C-PAF was simultaneously applied with EGF to search for additive effects. However, as shown in Fig. 2C, PAF completely suppressed the EGF-induced mitogenic response. C-PAF also suppressed this response, and was about a hundred times as effective as PAF (Fig. 2D). PAF or C-PAF had no apparent effects on wild type NRK cells. When a PAF antagonist, WEB 2086 (10 µM) or BN50730 (1 µM), was added together with PAF or C-PAF, both stimulatory and inhibitory effects disappeared (data not shown), thereby indicating that all these effects were receptor-mediated and not due to nonspecific actions. Because C-PAF is slightly less potent than PAF regarding receptor binding of NP3 cells (data not shown), its metabolic stability might explain the effects different from those of PAF. Since PAF is rapidly inactivated by PAF acetylhydrolase in serum (37–39) and cytosol (40), its effect is relatively transient at low concentrations. Since C-PAF is resistant to enzymatic inactivation by virtue of the methylcarbamyl moiety at its sn-2 position, its half-life is much longer than that of PAF. We therefore attempted to restrict the exposure of cells to C-PAF to a limited period of time by neutralizing it with WEB 2086. In Fig. 3, EGF, C-PAF, and WEB 2086 were applied sequentially at designated intervals. The addition of WEB 2086 3 h after C-PAF addition resulted in mitogenic activation (Fig. 3, column 3), an event not observed at any concentrations of C-PAF in the experiments shown in Fig. 2B. When WEB 2086 was added 6 h after C-PAF addition, this positive mitogenic

FIG. 3. Restriction of exposure time of the cells to C-PAF by a PAF antagonist WEB 2086. The mitogenic responses of NP3 cells were examined under various conditions when C-PAF (10 nM), PAF antagonist WEB 2086 (1 µM), and EGF (5 ng/ml) were added separately at designated times (indicated in hours). 1, control; 2–4, C-PAF was added at 0 time followed by WEB 2086 with 0–6 h intervals; 5, C-PAF alone; 6, EGF alone; 7–12, EGF and C-PAF were added at 0 time followed by WEB 2086 with 0–10 h intervals; 13–15, EGF was added at 0 time followed by C-PAF with 0–10 h intervals. The values are the averages of triplicate cultures and expressed as in Fig. 2. The vertical bars show the standard errors of the mean.
response was no longer observed (columns 4 and 5). Furthermore, C-PAF induced an additive mitogenic response to EGF, when WEB 2086 was added within 4 h after the addition of C-PAF (columns 8 and 9). Delayed addition of WEB 2086 after 6 h resulted in the manifestation of the inhibitory effect of C-PAF (columns 10–13). The addition of C-PAF 4 h after EGF-stimulation was still inhibitory (column 14), but was not inhibitory after 10 h (column 15). The reversal point from stimulation to inhibition was about 4 h after addition of the growth factor, therefore PAF induced different responses at different phases of the cell cycle. Under the conditions we used, NRK cells began to enter S phase around 8–10 h and the phase completed about 16–18 h after the stimulation, as observed by pulse [3H]thymidine uptake labeling and flow cytometric analyses (35). This means that PAF drives G0-arrested cells to proceed through the G1 phase, but arrests them before entry into S phase. It is, therefore, not the concentration of PAF but the phase of the cell cycle that determines the dual response to PAF.

Effect of PTX Treatment—Because PAFR is shown to couple with at least 2 types of G proteins, PTX-sensitive and -insensitive (16, 26), we treated NRK cells with PTX. Preincubation with 10 ng/ml PTX for 24 h, which inactivates Gt and Gq proteins, abolished the growth stimulatory effects of PAF, but the responses to EGF were not affected (Fig. 4). PTX treatment did not influence the inhibitory activity of C-PAF against EGF. Thus PTX-sensitive components, probably Gt, might participate in PAF-induced growth stimulatory responses in NRK cells, while PTX resistant components, probably Gq function in inhibitory responses.

Effects on Anchorage-dependent and Independent Growth—Because only one cell cycle can be monitored by the [3H]thymidine uptake assay, long-term effects of PAF on cellular growth were examined by the XTT assay, as described under “Materials and Methods.” As shown in Fig. 5, B and C, C-PAF had no detectable effects on PAFR expressing cells grown in regular media supplemented with 5% FCS, but did completely block EGF-induced growth acceleration. The inhibitory effects of C-PAF were abolished by the PAF antagonist WEB 2086, and were not observed in wild type cells (Fig. 5A). We detected no growth stimulatory effects in this assay.

We next examined the effect of C-PAF on anchorage-independent growth, using poly-HEMA-coated plates (34). NRK cells do not attach to poly-HEMA-coated plates and thus failed to grow (Fig. 5D, open circle). The addition of EGF promotes growth without anchorage (Fig. 5D, closed circle). The addition of C-PAF suppressed EGF-induced anchorage-independent growth of NP3 and NP5 cells (Fig. 5, E and F, closed square), but not that of wild type NRK cells (Fig. 5D, closed square). The addition of C-PAF alone was without effect. We obtained similar results using conventional soft agar methods. C-PAF inhibited EGF-induced anchorage-independent growth in soft agar of PAFR overexpressing cells, but not of NRK cells (data not shown).

Suppression of Oncogenic Transformation—EGF not only induces mitogenic responses in NRK cells, but also changes growth characteristics from normal to transformed cells (41), resulting in accelerated growth rate, loss of contact inhibition, focus formation, and the potential for anchorage-independent growth. In addition, the EGF signal transduction pathway in

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**Fig. 5. Inhibition of EGF-induced growth acceleration and anchorage-independent growth by C-PAF.** NRK, NP3, and NP5 cells were seeded in 96-well microplates in Dulbecco’s modified Eagle’s medium with 5% FCS supplemented with none (○), 5 ng/ml EGF (●), 10 nM C-PAF (■), EGF and C-PAF (▲), EGF, C-PAF and 1 µM WEB 2086 (×), and their growth was monitored by XTT assay. A-C, cells were plated in regular microplates. D-F, cells were plated in poly-HEMA-coated microplates. The values are the averages of triplicate cultures.
NRK cells appears to be a main pathway utilized by oncogenes, including ras and src, as EGF-insensitive mutants are not transformed by these oncogenes (33, 35). As PAF inhibited the EGF signal, PAF may also inhibit oncogene signals. To further examine this point, we made use of activated oncogenes instead of EGF. NP3, NP5, and wild type NRK cells were transfected with retrovirus carrying either v-K-ras or v-src oncogene. Many foci appeared 4–5 days after transfection, and the cells isolated from the foci showed a phenotypical transformation. There was no difference in the number of foci or the morphology of the transformed cells in NP3, NP5, and NRK cells (data not shown). All the transformed cells showed prominent morphological changes, focus formations, and rapid growth in soft agar. Supplementation of 10 nM C-PAF reversed the shape changes (Fig. 6A) and strongly inhibited anchorage-independent growth (Fig. 6B) of the oncogene transformants of NP3 and NP5 cells, but not that of NRK cells. Wild type NRK transformants were insensitive to C-PAF treatment. Naturally-occurring PAF showed similar albeit weaker effects on v-K-ras and v-src transformants (data not shown).

**G1 Phase Cell Cycle Arrest of Oncogene Transformants by C-PAF**—Since C-PAF inhibited the EGF-induced mitogenic response at the G1 phase, as shown in Fig. 3, we examined the effect of C-PAF on the cell cycle, using flow cytometry. Rapidly growing v-K-ras and v-src transformants of NP3 cells were treated with 10 nM C-PAF and their cell cycles examined. As shown in Fig. 7, prior to C-PAF treatment, approximately 50% of the cells were in the G1 phase. However, after adding C-PAF, nearly 90% of the cells accumulated into the G1 phase within 24 h. These results, together with findings as shown in Fig. 3, indicate that C-PAF inhibits the oncogene-induced transformation phenotype, as well as the effects induced by EGF at the G1 phase of the cell cycle, through a receptor-mediated pathway.

**Inhibition of Cyclin D1 by C-PAF**—Since the inhibition by C-PAF targeted on the G1 to S transition (4–8 h after stimulation), we examined the expression of cyclin and cyclin-dependent kinases (Cdk) known to play critical roles in this transition (42). NP3 cells were growth-arrested by low serum, stimulated by EGF with or without C-PAF, and mRNA and protein were collected at various intervals. Western blots showed that Cdk2 and Cdk4 protein levels were not significantly changed by EGF or C-PAF treatment (data not shown). In contrast, cyclin D1 mRNA and protein accumulated and peaked at 4–6 h after EGF addition (Fig. 8, A and B). Simultaneous addition of C-PAF and EGF abolished the accumulation of both mRNA and protein. Similarly, the addition of C-PAF to the rapidly growing ras and src transformants resulted in a rapid decrease in cyclin D1 mRNA and protein (Fig. 8, C and D). The reduction of cyclin D1 protein correlated well with the inhibitory effects of C-PAF.

**DISCUSSION**

PAF is a phospholipid mediator with versatile biological activities. This mediator is involved in inflammatory processes and in reproductive, nervous, and cardiovascular systems (43). While several groups of investigators reported that PAF induces cell growth or differentiation of various types of cells (8,
9, 10, 44, 45), it was not evident whether these actions might involve the receptor-G-protein system, or nonspecific actions of polar lipids. The present study clearly demonstrated that PAF has dual regulatory effects, growth stimulatory and inhibitory, mediated through the cloned, seven transmembrane spanning PAFR, depending on phases of the cell cycle.

PAF induced mitogenic responses in NRK fibroblasts expressing PAFR. The dose-response curves of the stimulatory effect were bell-shaped (Fig. 2A), suggesting that high doses of PAF result in growth inhibitory effects. Because C-PAF, a metabolically stable analogue of PAF, cancelled out these stimulatory and inhibitory effects at all doses used (Fig. 2B), the bell-shape observed for PAF is not due to the concentration itself, but possibly due to the exposure time of cells to effective doses of PAF. Thus, by restricting the exposure time of cells to C-PAF using the PAF antagonist WEB 2086 (Fig. 3), we found that C-PAF is growth-stimulatory when present only in the first 3 h. The reversal point from stimulation to inhibition was around 4 h after addition of the growth factors (Fig. 3, columns 9 and 10). These observations are interpreted to mean that PAF acts in the opposite direction depending on phase of the cell cycle. As schematically illustrated in Fig. 9, PAF may drive G<sub>0</sub>-arrested cells into the G<sub>1</sub> phase, but inhibit cell cycle progression from G<sub>1</sub> to S (see below). Under physiological conditions, the growth stimulatory effect might be predominant, since PAF concentrations in vivo are very low, and PAF is rapidly inactivated by the PAF acetylhydrolase present in the serum and cells. We obtained in vivo evidence to support the idea by using transgenic mice overexpressing PAFR. In the skin of transgenic mice, proliferations of keratinocytes and melanocytes was abnormally enhanced and progression to skin tumors with aging sometimes occurred (29).

On the other hand, growth inhibitory effects of PAF are also important. PAF inhibited EGF-induced growth acceleration and anchorage independent growth (Fig. 5) and also reversed morphological changes and anchorage-independent growth of oncogene transformants (Fig. 6). These results suggest that PAF inhibited component(s) shared by both EGF and oncogenes.

The molecular mechanisms governing these opposite functions of PAF remain elusive. PAF exerts biological functions through both PTX-sensitive and -insensitive G-proteins (16). The PTX-sensitive pathway is responsible for growth stimulatory effects, and PTX-resistant pathway functions for inhibitory effects (Figs. 4 and 9). It is widely recognized that activation of G<sub>i</sub> proteins leads to cell growth; a constitutively active mutant G<sub>i</sub> protein was found to be oncogenic (46). The activation of G16, one of the G<sub>q</sub> family proteins, was found to be growth inhibitory (47). Taken together with our previous findings, these results suggest that PAFR activates both G<sub>i</sub> and G<sub>q</sub>, resulting in two opposing effects on cell growth. The downstream cellular components activated in response to these G proteins are not fully defined. However, as for growth inhibitory effects, our data do suggest a role for cyclins. Cyclin D1,
also known as PRAD1, is an oncogene isolated from pituitary adenoma cells (48), and is amplified in various types of cancers. Cyclin D1, an activator of Cdk2 and Cdk4, is induced by various growth stimuli or activated oncogenes (49), and is suppressed by growth inhibitory factors such as transforming growth factor-β (50). Overexpression of cyclin D1 accelerates the cell cycle resulting in cellular transformation (51). The overexpression of Cdk activator CDC25 and inhibitor INK4 were reported to be transforming and suppressive to transformation, respectively (52, 53). Thus, it is conceivable that PAF inhibits the mitogenic response and transformation phenotypes by altering cyclin D1 levels. We entertain the notion that the PAF-PAFR system may possibly be used as a tool to investigate the mechanism of cell growth and oncogenic transformation. PAF or its metabolically-stable analogue which suppresses oncogenic transformation, may even prove to be a new type of anti-tumor drug.

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