

The Platelet-Activating Factor Receptor Activates the Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase and Induces Proliferation of Epidermal Cells through an Epidermal Growth Factor-Receptor-Dependent Pathway

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ABSTRACT

Platelet-activating factor (PAF) is a lipid mediator that has been implicated in a variety of keratinocyte functions. Keratinocytes express the specific receptor for PAF (PAF-R), a seven-transmembrane G-protein-coupled receptor. Although PAF-R-dependent stimulation of numerous signal transduction pathways has been shown in a variety of cell types, to date there has been no analysis of PAF-R signal transduction in human epidermal cells. There is also contradictory evidence that PAF acts as either a suppressor or activator of keratinocyte proliferation. Using a model system created by retroviral-mediated transduction of the PAF-R into the PAF-R-negative epidermal cell line KB, we now demonstrate that the activation of the epidermal PAF-R results in the activation of both the extracellular signal-regulated kinase (ERK) and p38, but not the jun N-terminal kinase mitogen-activated protein (MAP) kinase pathways. Ad-

ditionally, we show that the activation of the PAF-R stimulates the replication of epidermal cells. The activation of the ERK signal transduction pathway, as well as the PAF-dependent increase in cell proliferation, was dependent on the transactivation of the epidermal growth factor receptor (EGF-R). PAF-R-induced transactivation of the EGF-R was blocked by pharmacologic inhibitors of matrix metalloproteinases, of heparin-binding epidermal growth factor (HB-EGF), and specific inhibitors of the EGF-R tyrosine kinase. Activation of p38 MAP kinase by the PAF-R was not dependent on EGF-R activation and represents a distinct pathway of PAF-R-mediated signal transduction. In summary, these studies provide a mechanism whereby the PAF-R can exert proliferative effects through the activation of the EGF-R.

Platelet-activating factor (1-*O*-alkyl-2-acetyl glycerophosphocholine; PAF) is a mediator derived from glycerophosphocholine, which has been implicated in pathophysiological processes ranging from parturition to sepsis (Hanahan, 1992; Pinckard et al., 1994). Although PAF can be metabolized

to other biologically active lipids (Wilcox et al., 1987), the majority of PAF effects are probably due to its interaction with a specific G-protein-coupled transmembrane receptor (PAF-R; for review, see Izumi and Shimizu, 1995; Ishii and Shimizu, 2000). The activated PAF-R is linked to numerous signal transduction pathways, including phospholipase A₂, phospholipase C, phospholipase D, MAP kinase cascades, and adenylate cyclase. The end results of these signal transduction pathways can be as diverse as the range of factors

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ABBREVIATIONS: PAF, platelet-activating factor; PAF-R, PAF receptor; CPAF, 1-*O*-hexadecyl-2-*N*-methylcarbamoyl-glycerophosphocholine or carbamoyl PAF; EGF-R, epidermal growth factor receptor; HB-EGF, heparin-binding EGF; DAPH, 4,5-dianilinophthalimide; GPCR, G-protein-coupled receptor; MMP, matrix metalloproteinase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, jun N-terminal kinase; PI 3'K, phosphatidylinositol 3-kinase; PKC, protein kinase C; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PD98059, 2'-amino-3'-methoxyflavone; WEB-2086, morpholine,4-(3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f)(1,2,4)triazolo(4,3-a)(1,4)diazepin-2-yl)-1-oxopropyl; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; AG 1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; GM6001, *N*-(2*R*)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide; CRM 197, [Glu⁵²]-diphtheria toxin.

that are activated, including proinflammatory and trophic effects.

In the skin, there is evidence that PAF-mediated pathways are involved in cutaneous inflammation and keratinocyte stress responses. Although not found in normal skin, PAF is detected in inflammatory skin disorders such as urticaria, immunobullous diseases, and psoriasis (Grandel et al., 1985; Mallet and Cunningham, 1985; Travers et al., 1998). Furthermore, an intradermal injection of PAF induces a wheal and flare reaction (Henocq and Vargaftig, 1986; Travers et al., 1998). Keratinocytes synthesize PAF and related 1-acyl PAF-like species in response to various stimuli including ionophores, growth factors, PAF agonists, the pro-oxidative stressor *tert*-butyl hydroperoxide, ultraviolet light irradiation, and acute thermal damage (Sheng and Birkle, 1995; Travers, 1999). Activation of the PAF-R in keratinocytes leads to the production and release of PAF, IL-6, IL-8, tumor necrosis factor α , and the inducible form of cyclooxygenase (COX-2) (Pei et al., 1998; Dy et al., 1999). Reports describing the influence of the PAF-R on keratinocyte proliferation up to now have been conflicting. When transgenic mice over-express the PAF-R, they spontaneously develop areas of cutaneous inflammation and epidermal acanthosis, which is indicative of keratinocyte hyperproliferation (Ishii et al., 1997; Sato et al., 1999). Paradoxically, the cells that predominantly express the PAF-R in human epidermis are postmitotic suprabasal keratinocytes (Travers et al., 1995), and other investigators have reported that PAF inhibits the growth of cultured human keratinocytes (Shimada et al., 1998). Therefore, there is a conflicting data that describe the role of PAF in keratinocyte proliferation.

Activation of the PAF-R leads to a myriad of signal transduction pathways, including protein kinase C, phosphatidylinositol 3-kinase (PI 3'K), protein tyrosine kinases, phospholipases, and MAP kinases (Ishii and Shimizu, 2000). To date, the PAF-R has been primarily associated with ERK and p38 MAP kinase activation in a variety of tissues, but the activation of JNK MAP kinase has been reported in hippocampal neurons (DeCoster et al., 1998). Interestingly, PAF-R-mediated ERK and p38 activation can differ according to the cell type and species in which the receptor is located. In Chinese hamster ovary cells, PAF was reported to activate ERK through a protein kinase C-dependent, Ras-independent pathway (Honda et al., 1994). On the contrary, in human neutrophils PAF stimulated ERK activation via MEK1/2, a downstream target of Ras (Coffer et al., 1998).

In this article, we report that in epidermal cells, activation of the PAF-R results in the activation of ERK and p38, but not JNK MAP kinases. Furthermore, PAF-R activation resulted in ERK-dependent cell proliferation. ERK activation by the PAF-R requires the cleavage of membrane-bound heparin-binding epidermal growth factor (HB-EGF) by matrix metalloproteinases (MMP) and the subsequent activation of the EGF receptor. However, activation of p38 by the PAF-R occurs through a distinct pathway.

Materials and Methods

Reagents. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. DAPH and AG 1478 were purchased from Sigma/RBI (Natick, MA). PD98059 was purchased from Calbiochem (San Diego, CA). The PAF-R antagonist WEB-2086

was kindly provided by Boehringer Ingelheim (Ridgefield, CT), and A-85783 was a gift from Dr. James Summers (Abbot Pharmaceuticals, Abbott Park, IL).

Cell Culture. KB, A-431, and HaCaT cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) as previously described (Barber et al., 1998). The KBPAF-R model system was created by transduction of PAF-R-negative KB cells with the MSCV2.1 retrovirus encoding the human leukocyte PAF-R as previously described (Pei et al., 1998; Travers et al., 1998). KB cells transduced with the PAF-R (KBP) or with control MSCV2.1 retrovirus (KBM) were characterized by Southern and Northern blot analyses, by radioligand binding assays, and by calcium mobilization studies to demonstrate that the PAF-R was functional (Pei et al., 1998).

Cell Proliferation. Cellular proliferation was measured using two distinct methods. 1) 1.0×10^5 cells were seeded onto 10-cm culture dishes. Cells were serum-starved for 24 to 48 h prior to experiments. Cells were treated with serum-free medium alone or medium containing 20 ng/ml EGF or 100 nM CPAF for 48 h. After treatment cells were harvested, cell number was determined using a Coulter counter (Beckman Coulter, Inc., Fullerton, CA) with each treatment performed in duplicate. All experiments were replicated with at least two separate KBM and KBP clones. Mitogenic effects were analyzed using analysis of variance with Newman-Keuls post hoc tests. The significance for all tests was set at $p < 0.05$. 2) Cells were plated at a density of 5×10^3 cells/well in 96-well plates and allowed to stabilize for 1 day. Cells were serum-starved for 24 to 48 h prior to experiments. Cells were treated with serum-free medium alone or medium containing 20 ng/ml EGF or 100 nM CPAF for 48 h in the absence or presence of 5 or 50 μ M PD98059 or 1 or 10 μ M DAPH. Cell proliferation was determined using an MTT cell growth assay (Roche Applied Science, Indianapolis, IN) and analyzed using a microplate reader (Molecular Dynamics, Sunnyvale, CA) with optical density read at an absorbance of 570 nm. The effects of treatment are expressed as a percentage of viable cells using the untreated cells as the maximum cell viability. All experiments were performed in quadruplicate and replicated with at least two separate KBP clones. Mitogenic effects were analyzed using analysis of variance with Newman-Keuls post hoc tests. The significance for all tests was set at $p < 0.05$.

MAP Kinase Assays. Activation of ERK and p38 MAP kinase was determined through the immunoblotting of epidermal cell lysates using antibodies that specifically recognize the phosphorylated amino acid residues on activated phospho-ERK and phospho-p38. Following the indicated treatment of each cell type, KBM or KBP cells were washed twice with ice-cold phosphate-buffered saline and lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40) containing 0.5 mM Pefabloc SC (Roche Applied Science), and 10 mM sodium orthovanadate for 20 min on ice. The cell lysates were then harvested from the culture dishes, and the cellular debris was removed by centrifugation. Of the total cell lysates 40 μ m were separated on a 10% denaturing polyacrylamide gel, and the proteins were subsequently transferred to Immobilon-P (Millipore, Bedford, MA) membranes. Activated ERK protein was detected using α -phospho-ERK antibodies (New England BioLabs, Beverly, MA), whereas activated p38 was detected using α -phospho-p38 antibodies (New England BioLabs), followed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Total ERK and p38 proteins were detected on stripped immunoblots that were re-probed with antibodies to ERK and p38 (New England BioLabs).

EGF-R Activation. The activation of the EGF-R was assayed from epidermal cell lysates. KBM and KBP cells were lysed as described above and the EGF-R was immunoprecipitated from cell lysates by incubation with a polyclonal α -EGF-R antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Protein G Plus/Protein A agarose (Calbiochem) overnight at 4°C. Specific tyrosine phosphory-

lation on the immunoprecipitated EGF-R was determined by immunoblotting with α -phosphotyrosine antibodies (Transduction Laboratories, Lexington, KY) and enhanced chemiluminescence (Amersham Biosciences). To determine the total amount of EGF-R immunoprecipitated, the immunoblots were stripped of α -phosphotyrosine antibodies and reprobred with α -EGF-R antibodies.

Results

Stimulation of the PAF-R Activates ERK and p38. To determine which MAP kinase signal transduction pathways were activated in human epidermal cells, we utilized a model system constructed to specifically examine the effects of the PAF-R on epidermal cells. KB is an epithelial cell line originally derived from a nasopharyngeal carcinoma that does not express PAF-Rs (Pei et al., 1998). KB cells were infected with a retrovirus that contained the PAF-R cDNA (KBP cells; PAF-R⁺) or with a retrovirus that contained only the empty viral backbone (KBM cells; PAF-R⁻). The expression and function of the PAF-R in these cells have been confirmed previously (Barber et al., 1998; Pei et al., 1998; Travers et al., 1998). KBM and KBP cells were grown in serum-free media for 18 h, then treated with the metabolically stable PAF-R agonist CPAF, and the cells were harvested at the indicated times following stimulation (Fig. 1; previous experiments have determined that 100 nM CPAF elicits the optimum biologic response, J. B. Travers, unpublished data). The cell lysates were then analyzed for the active forms of ERK, p38, and JNK. The expression of total ERK, p38, and JNK protein

was also determined on the same immunoblot. No activated p38 or JNK proteins were seen in KBM cells following CPAF treatment, despite abundant levels of total p38 protein and total JNK protein (Fig. 1 and data not shown). A small increase in activated ERK protein was seen in KBM cells following CPAF, which was the same effect observed when only the ethanol vehicle was added to KBM cells (data not shown). In contrast to KBM cells, both activated p38 and ERK were seen in KBP cells within 6 min of CPAF treatment. The activation of p38 was more transient than the activation of ERK, with activated p38 appearing within 6 min and disappearing by 30 min, whereas activated ERK was observed for a more sustained period of time, that is up to 4 h after CPAF treatment. No activated JNK protein was found in KBP cells following CPAF treatment, yet UVB treatment resulted in activated JNK protein (data not shown). To ensure that the ERK signaling pathway was functioning the same in both KBM and KBP cells, the cells were serum-starved for 18 h and then stimulated with EGF. Once again, cell lysates were prepared at various times following EGF treatment. As shown in Fig. 1, the activation of ERK following EGF stimulation was identical in KBM and KBP cells. Therefore, activation of the PAF-R led to stimulation of the p38 and ERK signal transduction pathways, but did not affect the JNK signaling pathway.

PAF-R Activation of ERK Mediates Epidermal Cell Proliferation. Since ERK activation is frequently part of the mitogenic signal transduction pathway, we sought to

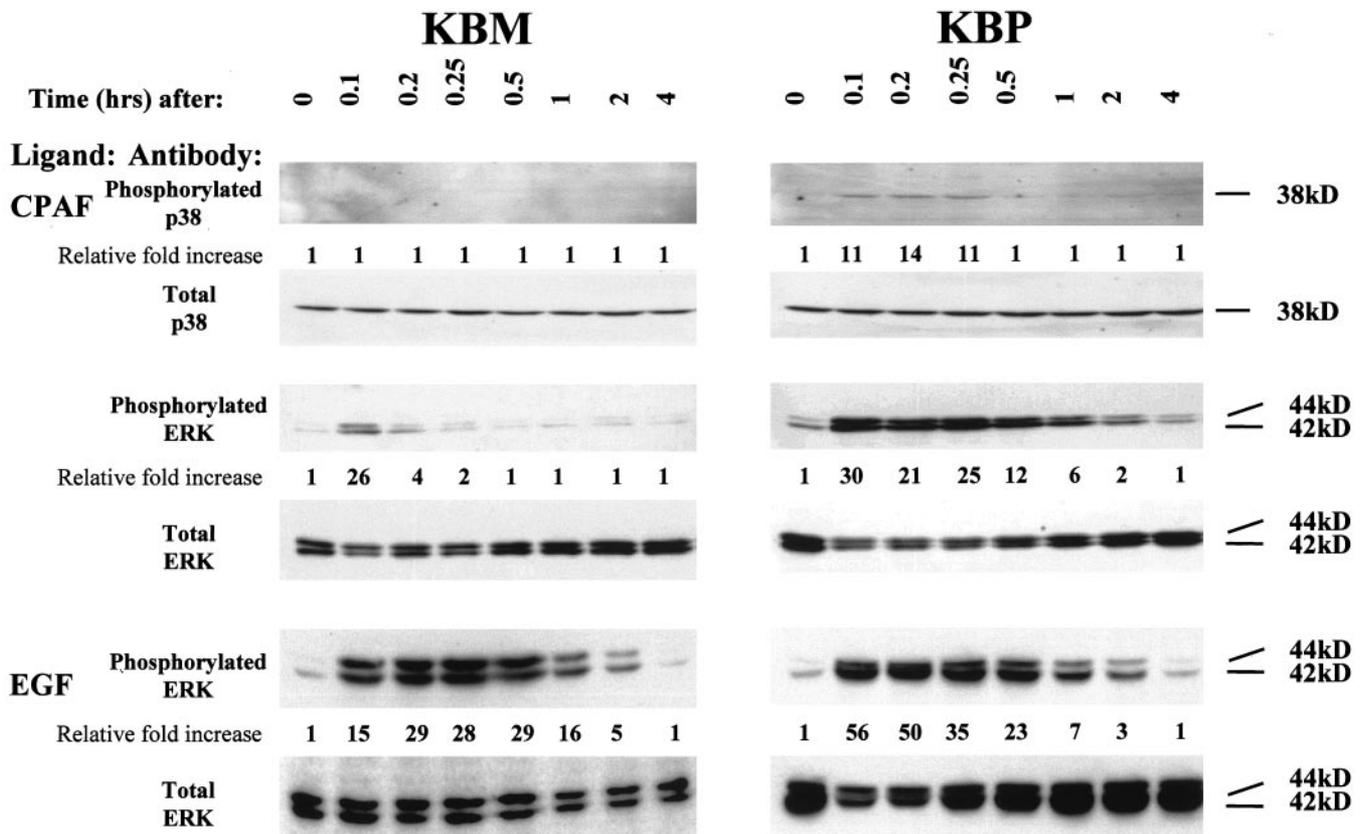


Fig. 1. The PAF-R activates p38 and ERK MAP kinase in epidermal cells. KBM cells (PAF-R⁻) and KBP cells (PAF-R⁺) were serum-starved for 18 h and then treated with EGF (0.9 ng/ml) or CPAF (100 nM). At the indicated time following CPAF or EGF treatment, cell lysates were prepared and assayed for phosphorylated MAP kinase proteins as described under *Materials and Methods*. The immunoblots were then stripped and reprobred with antibodies that recognize total MAP kinase protein. The relative fold increases in p38 and ERK phosphorylation were determined by scanning densitometry of autoradiographs.

determine whether the PAF-R-induced cellular proliferation. The potent mitogenic stimulator EGF was used as a positive control for proliferation (Ishii et al., 1997; Budillon et al., 1999; Sato et al., 1999). Exposure to 20 ng/ml EGF for 48 h significantly increased the proliferation of both KBP and KBM cells (Fig. 2A), confirming studies by Budillon and coworkers (1999) that demonstrated the proliferative role of EGF in paternal KB cells. To address whether PAF would result in a similar increase in cell number, KBM and KBP cells were treated with 100 nM CPAF for 48 h and then epidermal cell proliferation was assessed. Exposure to CPAF significantly increased the proliferation of KBP cells but had no effect on the proliferation of KBM cells (Fig. 2A), indicating that activation of the PAF-R likely mediates the CPAF-induced proliferation. Since activation of either PAF-R or EGF-R results in the activation of ERK in KBP cells (Fig. 1), we examined whether the inhibition of ERK would abolish the proliferative effects of CPAF and EGF. KBP cells were treated with the MEK inhibitor, PD98059 (5 and 50 μ M), for 30 min prior to and throughout the 48-h exposure to EGF or CPAF. As shown in Fig. 2B, inhibition of the ERK pathway abolished the proliferative effects induced by both PAF-R and EGF-R agonists. No inhibition of proliferation was observed when only the dimethyl sulfoxide vehicle was added to EGF- or CPAF-treated KBP cells (data not shown). To verify the effects of PD98059 on ERK phosphorylation, KBP cells were treated with CPAF or EGF in the presence or absence of 50 μ M PD98059. Cells were harvested, and the cell lysates were

then analyzed for the active forms of ERK. Both the CPAF- and EGF-induced ERK activation were abolished by PD98059 (Fig. 2C). These studies suggest that activation of the PAF-R results in the subsequent activation of ERK, which mediates the PAF-induced proliferation of epidermal cells.

PAF-R Activation of ERK and p38 Involve the Activation of Distinct Pathways. In other cell systems, the activation of ERK or p38 kinase has been demonstrated to occur via the activation of PI 3'K or the EGF-R. To determine whether the PAF-R activated either ERK or p38 through this pathway, KBP cells were treated with one of two separate pharmacologic inhibitors of PI 3'K, wortmannin or LY294002 (Fig. 3), or two separate pharmacologic inhibitors of EGF-R tyrosine kinase activity, DAPH and AG 1478 (Fig. 4). As seen in Fig. 3, the addition of either LY294002 or wortmannin did not influence the activation of either ERK or p38 by CPAF. Therefore, it is unlikely that PI 3'K is involved in the PAF-R signaling pathway that leads to the activation of ERK or p38. Consistent with the lack of effects of PI 3'K inhibitors, CPAF treatment did not activate AKT in KBP cells (data not shown). In contrast, the EGF-R inhibitors DAPH and AG 1478 each reduced the PAF-R-induced activation of ERK by about 70% (Fig. 4). However, neither DAPH nor AG 1478 caused any inhibition of PAF-R-mediated p38 activation. These data indicate that the activation of ERK by the PAF-R is dependent on the tyrosine kinase activity of the EGF-R and

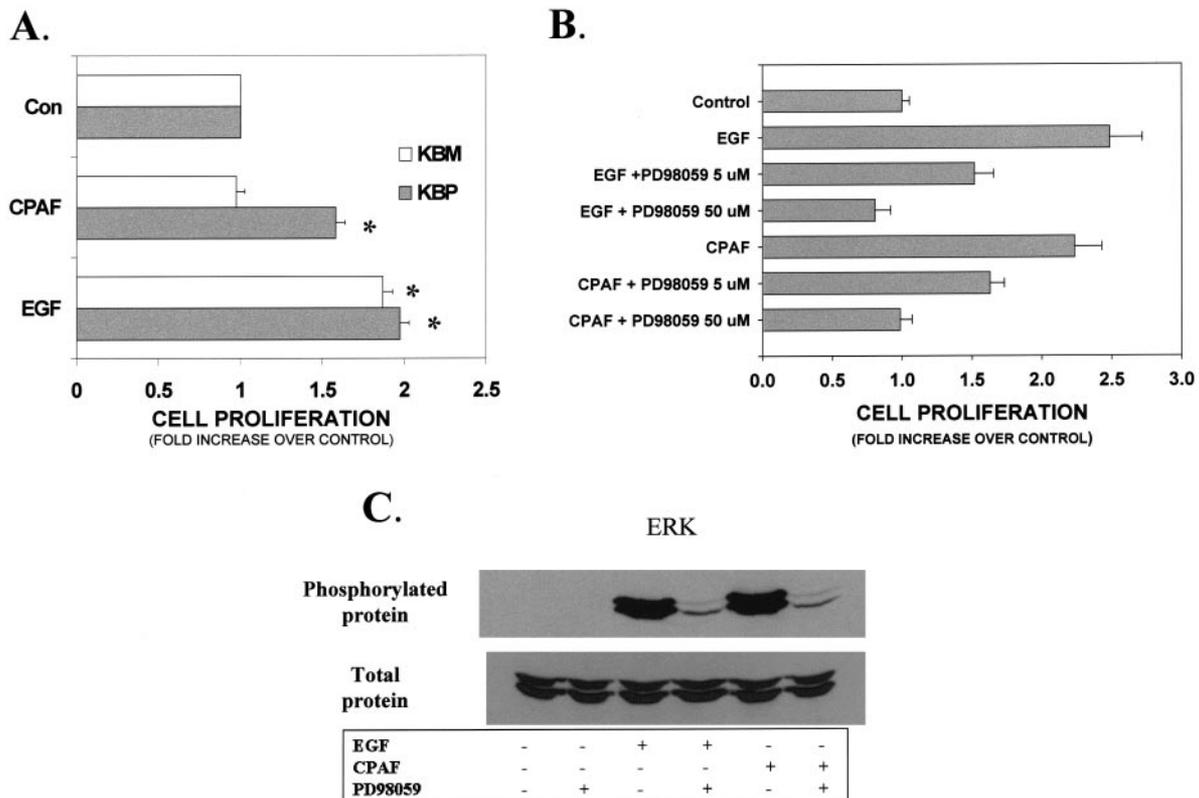


Fig. 2. CPAF induces cell proliferation only in KBP cells through an ERK-dependent mechanism. Cells were serum-starved for 24 to 48 h, and then incubated for 48 h in the presence of 100 nM CPAF or 20 ng/ml EGF, and proliferation was determined as an increase in cell number compared with unstimulated cells. A, KBM and KBP cells were treated with CPAF or EGF. B, KBP cells treated with CPAF or EGF in the presence or absence of 5 or 50 μ M PD98059. An asterisk indicates a significant difference from control using an analysis of variance with Newman-Keuls post hoc test ($p < 0.05$). C, KBP cells were pretreated with medium alone or medium containing 50 μ M PD98059. Following pretreatment, the KBP cells were stimulated with 100 nM CPAF or 0.9 ng/ml EGF and harvested after 20 min. The cell lysates were then assayed for either ERK activation.

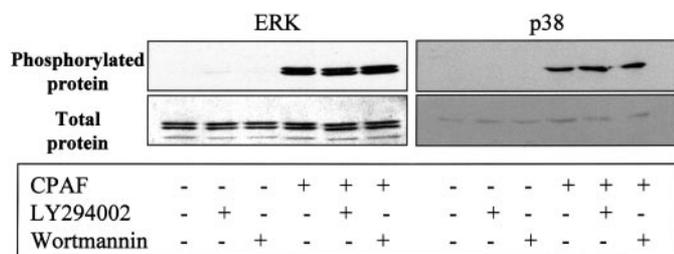


Fig. 3. PAF-R activation of ERK and p38 does not require PI 3'K. KBP cells were grown in serum-free medium overnight and then either sham-treated, treated with 0.1 μ M wortmannin, or treated with 10 μ M LY294002. Thirty minutes following the pretreatment with the PI 3'K inhibitors, the cells were left untreated or treated with 100 nM CPAF. The cells were harvested 20 min after the addition of CPAF and assayed for p38 and ERK MAP kinase activities.

that PAF-R-mediated ERK and p38 activation occurs through distinct mechanisms.

Effects of PAF-R Activation on EGF-R Tyrosine Phosphorylation. Since PAF-R-mediated activation of ERK could be influenced by inhibitors of the EGF-R tyrosine kinase, we sought to identify whether the PAF-R directly activated the EGF-R. Activation of the EGF-R, either through interaction with its ligand or via a ligand-independent process, results in dimerization and phosphorylation of tyrosine residues (Yarden and Schlessinger, 1987; Rosette and Karin, 1996; Moghal and Sternberg, 1999). To test whether the activation of the PAF-R influenced the activity of the EGF-R, KBP, or control, KBM cells were grown in EGF-deficient medium overnight and subsequently treated with CPAF or EGF as a control. At various times following treatment, the cells were lysed and assayed for tyrosine-specific phosphorylation of the EGF-R as described under *Materials and Methods*. The EGF-R in both KBM and KBP cells became activated in response to EGF stimulation; however, CPAF treatment resulted in EGF-R tyrosine phosphorylation only in KBP cells (Fig. 5). CPAF-induced EGF-R activation was seen within 6 min and was maximal between 6 and 60 min with levels returning close to baseline values by 4 h. To confirm that the activation of the PAF-R was required for CPAF-dependent EGF-R activation, KBP cells were pretreated with the PAF-R antagonist WEB-2086 prior to the addition of EGF or CPAF (Fig. 6). Inhibition of the PAF-R by 10 μ M WEB-2086 completely abolished the CPAF-dependent activation of the EGF-R in KBP cells. The specificity of the WEB-2086 compound for the PAF-R was demonstrated by the failure of WEB-2086 to influence EGF-dependent activation of the EGF-R. Similar inhibitory effects on CPAF- but not EGF-mediated EGF-R phosphorylation were found using 10 μ M the PAF-R antagonist A-85783 (data not shown). CPAF treatment of the PAF-R-positive human keratinocyte cell lines HaCaT and A-431 (Travers et al., 1995) also resulted in EGF-R phosphorylation and was inhibited by preincubation with PAF-R antagonists (data not shown). Therefore, cell signaling events as a consequence of activation of the epidermal PAF-R led to the tyrosine phosphorylation of the EGF-R.

PAF-R-Dependent Activation of the EGF-R Requires the Tyrosine Kinase Activity of the EGF-R. In other reported cases of EGF-independent activation of the EGF-R, the tyrosine phosphorylation was shown to be either independent (Yamauchi et al., 1997) or dependent (Daub et al.,

1996) on the native tyrosine kinase activity of the EGF-R. To determine whether the PAF-R-dependent activation of the EGF-R, and subsequently ERK activation, required the tyrosine kinase activity of the EGF-R, KBP cells were simultaneously treated with EGF or CPAF and specific inhibitors of the EGF-R tyrosine kinase, DAPH and AG 1478. As previously reported, pretreatment of cells with these tyrosine kinase inhibitors inhibited EGF-induced EGF-R tyrosine phosphorylation (Zhang et al., 1999) and ERK activation (Fig. 4). Preincubation of KBP cells with DAPH (Fig. 7A) or AG 1478 (Fig. 7B) inhibited CPAF-induced transactivation of the EGF-R in a dose-dependent fashion. To identify whether the transactivation of the EGF-R was required for PAF-R-induced cell proliferation, KBP cells were pretreated with DAPH (1 and 10 μ M) prior to stimulation with CPAF or EGF. The cellular growth of the KBP cells was then measured 48 h poststimulation. As seen in Fig. 7C, pretreatment of keratinocytes with DAPH eliminated the cell proliferation induced by CPAF or EGF treatment. These findings suggest that the majority of tyrosine phosphorylation of the EGF-R and cell proliferation observed following PAF-R activation was due to the intrinsic EGF-R tyrosine kinase.

PAF-R-Mediated Transactivation of the EGF-R Involves HB-EGF. Recent studies by Ullrich and colleagues have implicated MMP-mediated cleavage of HB-EGF as the mechanism by which some G-protein receptors can transactivate the EGF receptor (Prenzel et al., 1999). The following experiment was designed to test whether the epidermal PAF-R transactivates the EGF-R via this mechanism. As shown in Fig. 8, preincubation of KBP cells with the general MMP inhibitor GM6001 inhibited CPAF- (A) but not EGF-induced (B) EGF-R tyrosine phosphorylation. Similarly, the toxin CRM 197, which specifically binds to and inactivates HB-EGF (Mitamura et al., 1995), also inhibited CPAF- (Fig. 8A), but not EGF-induced (Fig. 8B) EGF-R activation. Finally, a monoclonal antibody directed against the extracellular portion of the EGF-R inhibited EGF-R phosphorylation induced by either EGF or CPAF (Fig. 8, A and B). Similar concentrations of an antibody directed against the cytosolic portion of the EGF-R did not appreciably inhibit EGF-R phosphorylation in response to these stimuli (Fig. 8A, control antibody). These studies suggest that PAF-R-mediated EGF-R transactivation involves MMP-mediated HB-EGF cleavage.

PAF-R-Mediated Activation of ERK is Dependent on MMP-Directed Cleavage of HB-EGF and Its Subsequent Activation of the EGF-R. We have shown that the PAF-R activates both ERK and p38 MAP kinases in epidermal cells. The activation of ERK by the PAF-R is dependent on the activity of the EGF-R tyrosine kinase, and the PAF-R activates the EGF-R through the MMP-dependent cleavage of HB-EGF. Therefore, it was necessary to determine whether the activation of ERK by the PAF-R was also dependent on the MMP-directed cleavage of HB-EGF. KBP cells were treated with CPAF in the presence of GM6001, CRM 197, a neutralizing EGF-R antibody, or an antibody to an intracellular epitope of the EGF-R. The activation of ERK or p38 was then determined following each of the treatments described. As stated previously, treatment with CPAF led to the activation of p38. However, the PAF-R-mediated activation of p38 was not affected by any of the indicated treatments (Fig. 9). CPAF treatment also led to the activation of

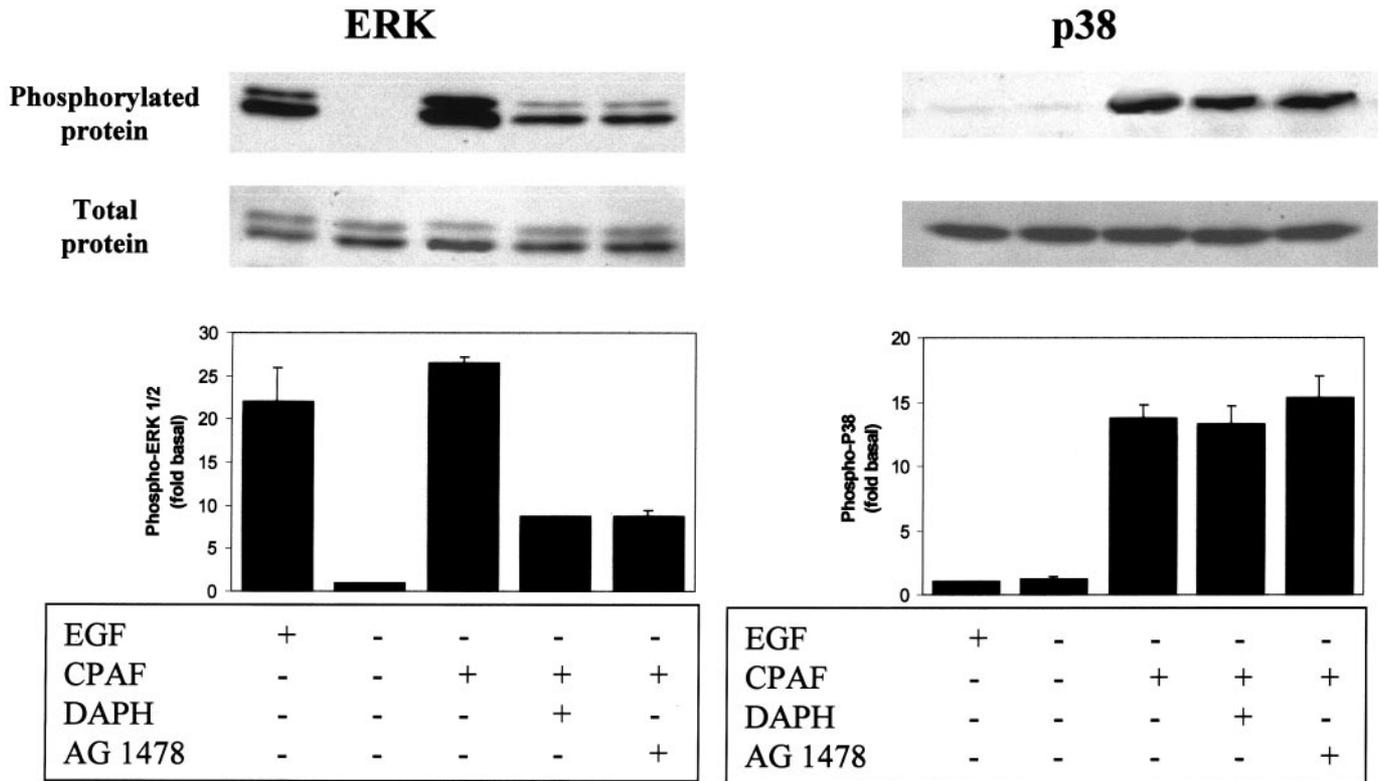


Fig. 4. Inhibitors of the EGF-R tyrosine kinase suppress CPAF-mediated ERK activation but not p38 activation. KBP cells were serum-starved overnight, then pretreated with either 0.1 μ M AG 1478 or 20 μ M DAPH for 20 min. The cells were subsequently treated with 100 nM CPAF or 0.9 ng/ml EGF for 20 min. At that time, the cells were harvested, and the cell lysates were assayed for p38 and ERK MAP kinase activity. The relative fold increases in p38 and ERK phosphorylation were determined by scanning densitometry of autoradiographs. The bar graph represents the average of three independent experiments.

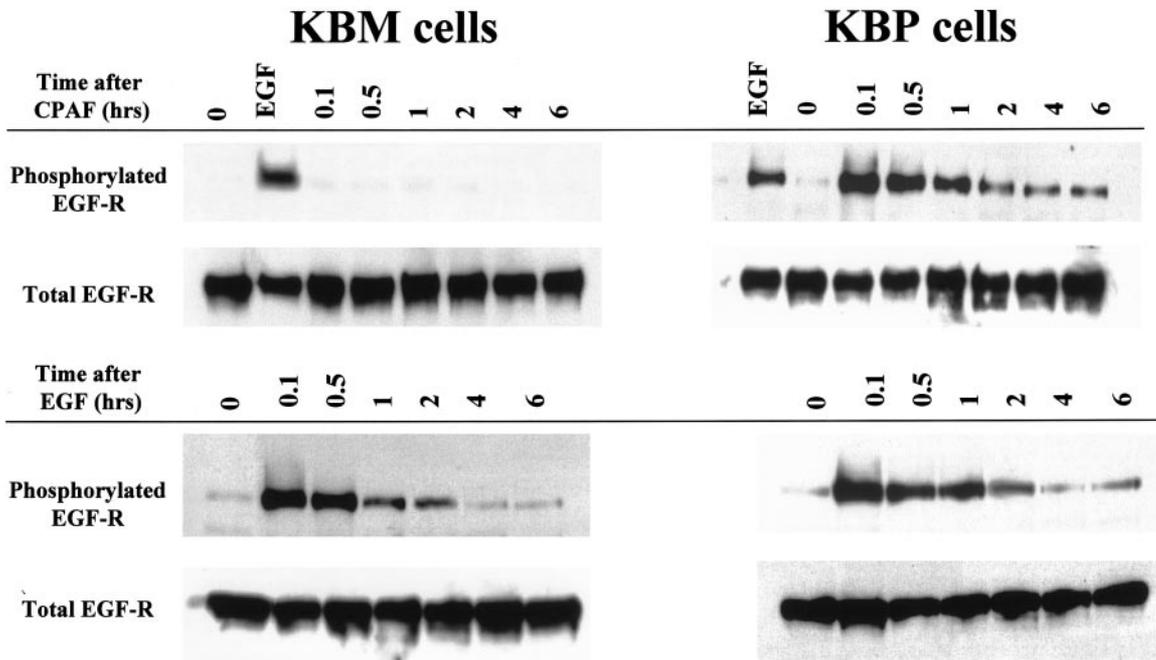


Fig. 5. The EGF-R is activated by CPAF in KBP cells, not in KBM cells. KBM and KBP cells were assayed for the induction of EGF-R tyrosine phosphorylation following the addition of CPAF as described under *Materials and Methods*. EGF-R tyrosine phosphorylation after EGF treatment was used as a control to ensure that the EGF-R was equally functional in both cell types.

ERK; however, in contrast to p38 activation, cotreatment of KBP cells with CPAF and GM6001, CRM 197, or neutralizing α -EGF-R antibodies inhibited the PAF-R-mediated activa-

tion of ERK (Fig. 9). Treatment of KBP cells with a non-neutralizing α -EGF-R antibody had no effect on PAF-R-induced ERK activation. These data imply that PAF-R-

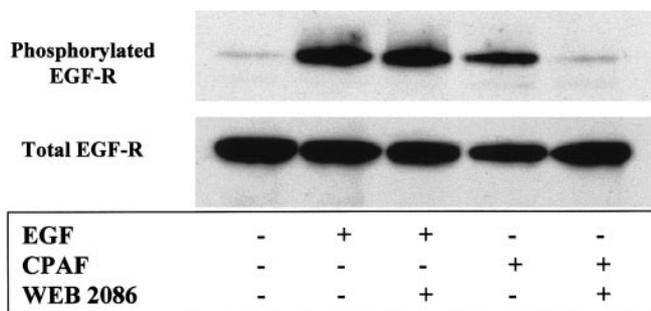


Fig. 6. The PAF-R is required for CPAF-mediated activation of the EGF-R. KBP cells were serum-starved overnight, then treated with the PAF-R antagonist WEB-2086 (10 μ M) for 30 min prior to the addition of either 0.9 ng/ml EGF or 100 nM CPAF. Cell lysates were assayed for EGF-R activation 20 min later.

dependent activation of ERK is controlled by MMP-directed cleavage of HB-EGF. The cleaved HB-EGF is then able to bind and activate the EGF-R, leading to the activation of ERK and subsequently cell proliferation. In contrast, PAF-R-induced p38 activation is not activated by this pathway or through a PI 3'K-mediated mechanism.

Discussion

In this article, we have demonstrated that the PAF-R is capable of stimulating the activation of ERK and p38 MAP kinase in epidermal cells, but does not influence the activation of the JNK MAP kinase. PAF-R-mediated ERK activation appears to be secondary to EGF-R stimulation since the EGF-R tyrosine kinase inhibitors, DAPH and AG 1478, blocked CPAF-induced ERK activation. In contrast to ERK activation, neither DAPH nor AG 1478 had any effect on the CPAF-induced p38 activation, suggesting that p38 activation is not mediated by activation of the EGF receptor. As illustrated in our model (Fig. 10), the transactivation of the EGF-R by the PAF-R involves a matrix metalloproteinase-dependent cleavage and secretion of HB-EGF that activates the EGF-R and subsequently phosphorylates ERK. The activation of ERK then leads to epidermal cell proliferation. The transactivation of the EGF-R by other G-protein-coupled receptors (GPCR) is thought to be an important signaling pathway by which these receptor types induce both mitogenic as well as motogenic effects (Mitamura et al., 1995; Prenzel et al., 1999; Eguchi et al., 2000) and provides a mechanism for the proliferative effects of PAF in epidermal cells.

Several lines of evidence suggest that the PAF-R-induced EGF-R transactivation involves the matrix metalloproteinase-dependent cleavage and release of HB-EGF. PAF-R-induced EGF-R phosphorylation and ERK activation were abolished in the presence of the nonspecific MMP inhibitor GM6001, whereas CPAF-induced p38 activation was unaffected. In addition, the HB-EGF neutralizing toxin CRM 197 (Mitamura et al., 1995; Prenzel et al., 1999) attenuated the EGF-R transactivation by CPAF, thus implicating the release of HB-EGF in the EGF-R transactivation. Although GM6001 and CRM 197 diminished PAF-R-mediated EGF-R phosphorylation and ERK activation, these treatments did not block the EGF-stimulated EGF-R and ERK phosphorylation, indicating that the EGF-R-dependent activation was not affected. Finally, a reduction in CPAF-induced EGF-R phosphorylation and ERK activation was achieved by a

monoclonal antibody against the ligand binding domain of the EGF-R. However, no reduction was seen in the presence of another EGF-R antibody that recognizes an intracellular domain of the EGF-R, suggesting that the initial reduction resulted from an inhibition of EGF or HB-EGF binding to the EGF-R. Our results with the MMP inhibitor GM6001, the diphtheria toxin mutant CRM 197, and a neutralizing antibody to the EGF-R further support the hypothesis that activation of the PAF-R results in transactivation of the EGF-R through an autocrine- or paracrine-dependent manner. Recently, the MMP-dependent cleavage of HB-EGF has been shown to be responsible for EGF-R transactivation induced by various other GPCR (Prenzel et al., 1999; Eguchi et al., 2000), indicating that the PAF-R-mediated EGF-R transactivation is through a similar pathway. Interestingly, PAF has also been demonstrated to induce the expression of HB-EGF in human peripheral blood monocytes (Pan et al., 1995), although it is unclear whether this response exists in epithelial cells.

Although our results indicate that activation of the PAF-R transactivates the EGF-R through a matrix metalloproteinase, the metalloproteinase involved in the HB-EGF release remains unknown. Matrix metalloproteinases are a family of zinc-dependent peptidases that degrade extracellular matrix components. Currently, 20 members of the human MMP family have been identified (Ravanti and Kahari, 2000). In our experiments with CPAF-induced EGF-R activation, pretreatment with the MMP inhibitor, GM6001, abolished the PAF-R-mediated transactivation of EGF-R and ERK activation. GM6001 is a nonselective MMP inhibitor, which has been previously shown to inhibit the human metalloproteinases MMP-1, 2, 3, 8, and 9 (Galaray et al., 1994). Furthermore, activation of the PAF-R in epidermal cells results in an increase in the biosynthesis of MMP-9 (J. B. Travers, unpublished observations), which could suggest that this metalloproteinase may be involved in EGF-R transactivation. Recently, the metalloproteinase ADAM9/MDC9 was shown to mediate the release of HB-EGF from a kidney cell line (Izumi et al., 1998) through a mechanism involving protein kinase C (PKC). Treatment of KBP cells with phorbol esters stimulates PKC and induced EGF-R activation. EGF-R activation by phorbol esters could be abolished using a PKC inhibitor; however, PKC inhibitors had no effect on the CPAF-induced activation of EGF-R (data not shown). These data indicate that PKC-dependent metalloproteinases are not involved in PAF-R-mediated EGF-R transactivation. These conclusions are supported by studies that indicate that EGF-R transactivations by other GPCRs are also not mediated by the PKC pathway (Prenzel et al., 1999; Eguchi et al., 2000).

The ability of the PAF-R to transactivate the EGF-R was significant as this pathway was found to be responsible for PAF-R-mediated ERK activation and the subsequent induction of cell proliferation. Although the PAF-R can activate both ERK and p38 in epidermal cells, apparently the activation of these two MAP kinase pathways occurs through separate mechanisms. Activation of the PAF-R results in ERK and p38 stimulation in neutrophils (Nick et al., 1997), airway smooth muscle cells (Maruoka et al., 2000), and in Chinese hamster ovary cells transfected with the PAF-R (Zhang et al., 1999). In addition, stimulation of the PAF-R results in activation of JNK/stress-activated protein kinase in airway smooth muscle cells (Maruoka et al., 2000) but not in epider-

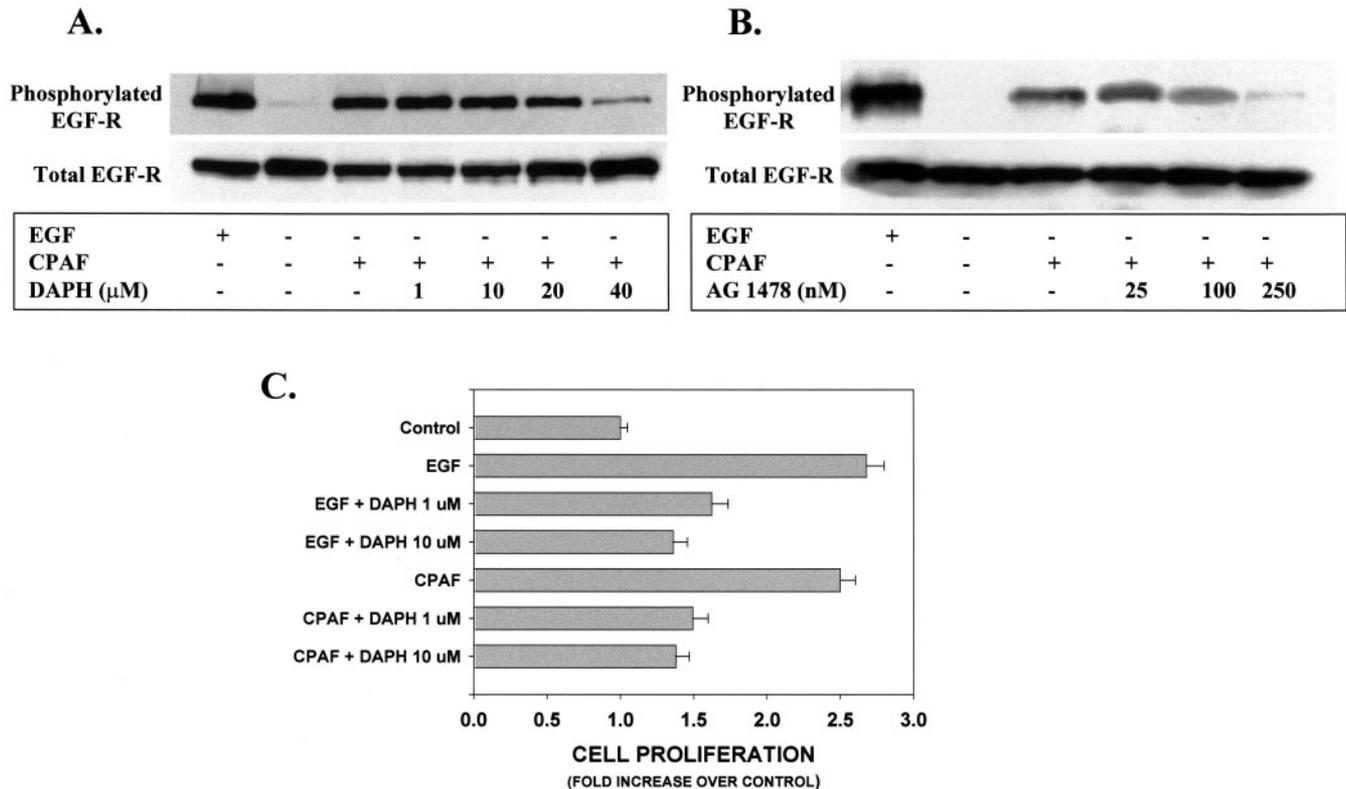


Fig. 7. PAF-R-mediated activation of the EGF-R and cell proliferation can be inhibited in a dose-dependent manner by inhibitors of the EGF-R tyrosine kinase. KBP cells were grown as described in Fig. 3 but were pretreated with the indicated increasing concentrations of either DAPH (A) or AG 1478 (B) prior to CPAF stimulation. The cells were harvested 20 min after the addition of CPAF and assayed for the activation of the EGF-R. Cells were also treated only with EGF as a control. C, KBP cells were grown in serum-free medium for 48 h and then pretreated with 1 or 10 μ M DAPH. The cells were stimulated with either EGF (20 ng/ml) or CPAF (100 nM) 30 min following DAPH treatment. Cell proliferation was assayed 48 h following growth stimulation using the MTT cell growth assay. The effects of treatment are expressed as a percentage of viable cells using untreated cells as the maximum cell viability.

mal cells stimulated with PAF agonists. Furthermore, activation of the EGF receptor with either CPAF or EGF did not induce activation of p38 (Figs. 1, 3, and 4). These results suggest that EGF receptor signaling is not homogenous among various cell lines. Indeed, activation of the EGF receptor in vascular smooth muscle cells (Eguchi et al., 2000) has been shown to induce activation of both ERK and p38 MAP kinases.

The results of our study indicate that PAF has mitogenic actions in epidermal cells and confirm previous findings that EGF induced proliferation in KB cells (Budillon et al., 1999). In fact, exposing KBP cells to EGF or CPAF significantly increased cell proliferation. Furthermore, the mitogenic actions of PAF appear to be mediated by the PAF-R since treatment with CPAF increased the proliferation of KBP but not KBM cells. These results are comparable to other reports of PAF-induced mitogenic actions; endogenous or exogenous administration of PAF induces an increase in cell proliferation in breast cancer cells that contain the PAF-R without affecting proliferation in PAF-R-negative breast cancer cells (Bussolati et al., 2000). These previous observations, when combined with our results, all suggest that the proliferative actions of PAF are dependent on the activation of the PAF-R. Other groups have reported that PAF-R-mediated proliferative responses in fibroblasts (Roth et al., 1996) or smooth muscle cells (Gaumond et al., 1997) can be blocked by tyrosine kinase inhibitors. These data support our model of the mechanism for the mitogenic effects of PAF. Thus, the trans-

activation of the EGF-R appears to be of critical importance for the mitogenic actions of GPCR. We examined whether the PAF-R-induced ERK activation resulted in proliferation since the ERK is the primary signaling pathway in growth factor-regulated proliferation (Davis, 1993) and PAF-R-induced ERK activation was secondary to transactivation of the EGF-R. Exposure to the MEK inhibitor, PD98059, inhibited the CPAF- and EGF-mediated activation of ERK (Fig. 2C) and abolished the CPAF- and EGF-induced proliferation of KBP cells (Fig. 2B). These results support the hypothesis that the PAF-induced increase in cell proliferation is mediated by transactivation of the EGF-R and subsequent activation of the ERK pathway (Fig. 10).

Recent studies have suggested that the PAF system plays an important role in keratinocyte function and stress responses. Human keratinocytes and epidermal cell lines do not synthesize significant amounts of PAF under resting conditions. However, numerous diverse stimuli including cytokines, UVB, physical damage, as well as PAF-R activation itself all result in significant levels of PAF biosynthesis in these cell types. Of note, these same stimuli result in an epidermal proliferative response. Keratinocytes also express functional PAF-Rs, and activation of the PAF-R in keratinocytes leads to the biosynthesis and release of numerous proinflammatory mediators, including IL-1, IL-6, IL-8, tumor necrosis factor, as well as release of PAF (Pei et al., 1998; Travers et al., 1998; Dy et al., 1999). Thus, the ability of the keratinocyte PAF-R to transactivate the EGF-R and subse-

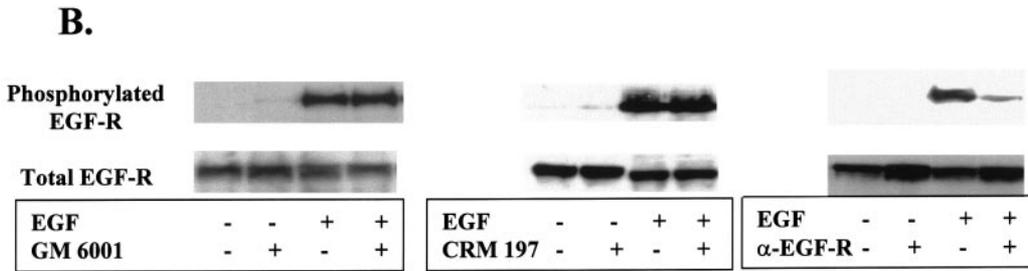
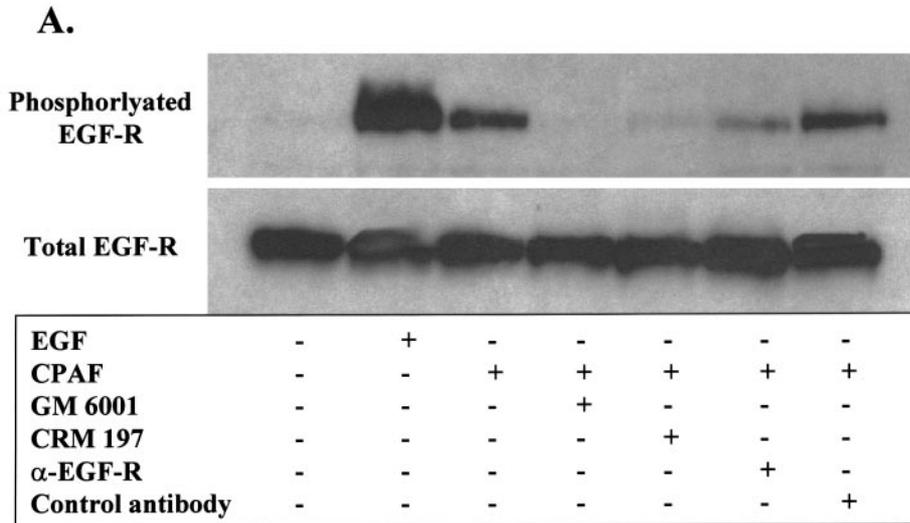


Fig. 8. PAF-R mediated activation of the EGF-R is dependent on MMP activity and the binding of HB-EGF to the EGF-R. A, KBP cells were grown overnight in serum-free medium. The cells were then either left unstimulated, pretreated with 10 μM GM6001 (general MMP inhibitor) for 60 min, pretreated with 10 μg/ml CRM 197 (specific inhibitor of HB-EGF), 10 μg/ml neutralizing α-EGF-R antibody, or 10 μg/ml non-neutralizing α-EGF-R antibody. Following pretreatment, the KBP cells were stimulated with 100 nM CPAF and harvested after 20 min. EGF-R activation was then determined from the cell lysates. B, KBP cells were treated as described in A, except that they were stimulated with 0.9 ng/ml EGF instead of CPAF.

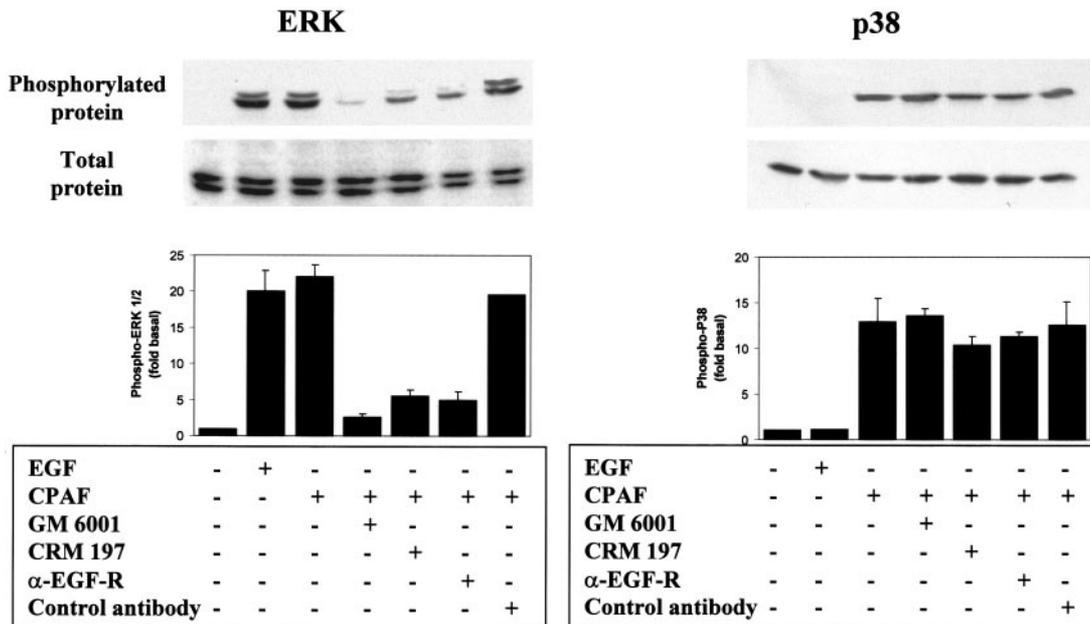


Fig. 9. PAF-R-mediated activation of ERK is dependent on MMP activity and the binding of HB-EGF to the EGF-R. KBP cells were treated as described in Fig. 7A, except the cell lysates were assayed for either ERK or p38 activation. The graphs shown represent fold increases in specific MAP kinase phosphorylation as determined by densitometric analyses of radiographs. The bar graph represents the average of three independent experiments.

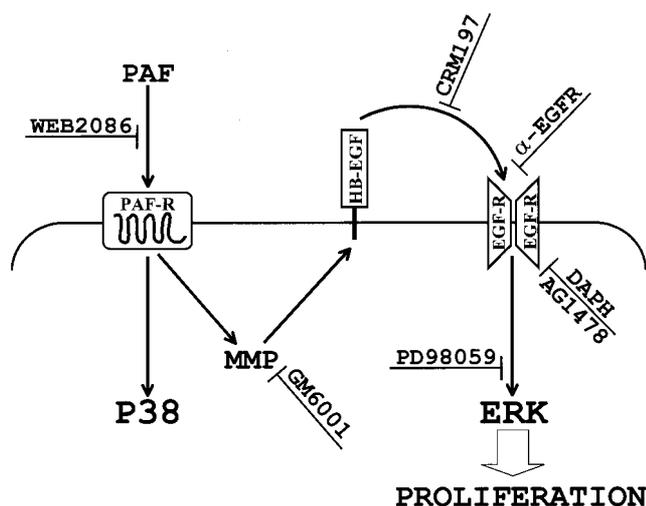


Fig. 10. Model of the proposed mechanism for PAF-R-mediated MAP kinase activation.

quently activate the ERK pathway appears to provide a mechanism for the PAF-R-mediated hyperproliferative dermatitis (Shimada et al., 1998; Sato et al., 1999). Further studies are warranted to determine whether PAF may be involved in epidermal proliferation that occurs during wound healing secondary to tissue injury or trauma.

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