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Platelet-Activating Factor Induction of Activator Protein-1 Signaling in Bronchial Epithelial Cells

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ABSTRACT

Platelet-activating factor (PAF) has been implicated in the pathogenesis of allergic and inflammatory events in the airway. In the present study, we sought to determine if PAF receptors are present on human bronchial epithelial cells and whether PAF binding to these receptors leads to activation of activator protein-1 (AP-1)-mediated transcription. Radioligand binding studies demonstrated specific binding sites for the PAF antagonist [^{3}H]WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-f]-[1,2,4]triazolo[4,3- *a*][1,4]diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) on primary bronchial epithelial cells with an equilibrium dissociation constant (K_d) = 9.8 nM and maximal density of binding sites (B_{max}) = 42.4 fmol/mg of protein. The expression of PAF receptors in these cells was further confirmed by reverse transcriptase-polymerase chain reaction, which revealed amplification products derived from PAF receptor mRNA corresponding to transcripts 1 and 2. In the bronchial epithelial cell line BEAS-2B transfected with an expression plasmid for the human PAF receptor, PAF stimulation increased AP-1 DNA binding activity as determined by electrophoretic mobility shift assays. The Fos and Jun family proteins were identified as components of the DNA-protein complexes by anti-peptide antibodies in gel supershift assays. Additionally, PAF significantly induced AP-1 mediated transcription which was dependent on the expression of PAF receptors. The PAF antagonist WEB 2086 blocked the PAF effect but not that induced by 12-*O*-tetradecanoyl phorbol-13-acetate, indicating the specificity of the PAF response. These results indicate that activation of airway epithelial cells through stimulation of PAF receptors includes up-regulation of the nuclear transcription factor AP-1 and AP-1 transcriptional activity.

PAF is a biologically active phospholipid that has been implicated in the pathogenesis of allergic and inflammatory events in the airway. Numerous studies have established an important potential role of PAF in airway inflammation (Chan-Yeung *et al.*, 1991; Kuitert and Barnes, 1995; Lee *et al.*, 1984). Possible effects of PAF on the lung epithelium are of particular interest, because bronchial epithelial cells are implicated in the pathophysiological changes of the lung and the amplification of airway inflammation (Levine, 1995). It has been demonstrated that bronchial epithelial cells produce a number of inflammatory cytokines (i.e., granulocyte-macrophage colony stimulating factor, regulated on activation normal T cell expressed and secreted, endothelin, IL-1, IL-6, IL-8, IL-10, IL-11, IL-16, transforming growth factor-β, tumor necrosis factor-α, prostaglandin E_2, and intercellular adhesion molecule-1) that have the capacity to recruit inflammatory cells into the airways and to activate these incoming inflammatory cells after their arrival (Levine, 1995). Thus, cytokines produced from bronchial epithelial cells could initiate and/or amplify inflammation in the airways. Whether PAF receptor activation is potentially involved in human bronchial epithelial cell activation is still unknown.

The actions of PAF are mediated mainly through specific cell surface receptors. The PAF receptor has seven putative transmembrane segments typical for a G protein-coupled receptor. A PAF receptor cDNA (transcript 1) has been cloned from guinea pig and human cells (Chase *et al.*, 1993; Seyfried *et al.*, 1992; Honda *et al.*, 1991) and was found ubiquitously, but most abundantly in peripheral leukocytes (Mutoh *et al.*, 1993). Recently, a PAF receptor cDNA with a different 5′-noncoding sequence (transcript 2) was isolated from human heart and has been found in lung, spleen, and kidney tissue, but not in leukocytes (Mutoh *et al.*, 1993). Interaction of PAF with its receptor activates a number of signaling pathways, including tyrosine kinases, phospholipase C, intracellular Ca^{2+} mobilization, and protein kinase C (Venable *et al.*, 1993). These PAF-mediated early biochemical events are of-

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ABBREVIATIONS: PAF, platelet-activating factor [(1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine)]; AP-1, activator protein-1; B_{max}, maximal density of binding sites; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; CAT, chloramphenicol acetyl transferase; NHBE, normal human bronchial epithelial; NF, nuclear factor; CMV, cytomegalovirus; RT, reverse transcriptase; IL, interleukin; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ten followed by the enhanced expression of genes for cytokines, such as IL-6, IL-8, and for specific adhesion molecules (Roth *et al.*, 1996; Albelda *et al.*, 1994). Also, PAF has been found to activate expression of the genes encoding collagenase type I, heparin-binding epidermal growth factor-like growth factor, NF-p50, mitogen-activated protein kinase, mitogen-activated protein kinase kinase, and immunoglobulin κ light chain in a wide variety of cell types (Honda *et al.*, 1994; Pan *et al.*, 1995; Bazan *et al.*, 1993; Smith and Shearer, 1994; Tan *et al.*, 1994). Transcriptional activation of the heparin-binding epidermal growth factor-like growth factor in response to PAF has been correlated with an increase in κB binding activity. Kravchenko *et al.* (1995) have recently shown that PAF activated κB binding activity in Chinese hamster ovary cells expressing the PAF receptor. These findings suggest an important function of PAF in the regulation of gene expression, although the transcription factors other than NF-κB involved in PAF-induced gene expression have yet to be characterized.

The transcription factor AP-1 is a complex comprised of a group of proteins encoded by the *jun* (c-Jun, JunB, JunD) and *fos* (c-Fos, FosB, Fra-1, and Fra-2) gene families, which can bind to the AP-1 consensus sequence either as Jun/Jun or Jun/Fos dimers (Angel and Karin, 1991). AP-1 activation has been studied in inflammatory and immunoregulatory cells; however, little is known about its activation by G protein-coupled receptors in human bronchial epithelial cells. In the present study, we sought to determine if PAF receptors are present on human bronchial epithelial cells and whether PAF binding to these receptors activates AP-1-mediated transcription.

Materials and Methods

Reporter and expression plasmids. The reporter plasmid p5xTRE-CAT was a generous gift from Dr. I. Verma (The Salk Institute, La Jolla, CA). Plasmid p5xTRE-CAT is a derivative of pBLCAT3 and has five AP-1 binding sites upstream of a TATA region linked to the CAT gene. The CMV-β-galactosidase construct contains the CMV promoter driving the expression of the β-galactosidase gene. The expression plasmid for the human PAF receptor (pBC12BI-PAFR) was created by cloning a 1090-bp *Hin*dIII-*Stu*I fragment containing the coding region for the human PAF receptor, into the eukaryotic expression vector pBC12BI (Stratagene, La Jolla, CA) (Chase *et al.*, 1993).

Cell culture and transfections. The BEAS-2B cell line was a generous gift from Dr. C. Harris (National Institutes of Health, Rockville, MD). These cells are a derivative of normal human bronchial epithelial cells explanted from large airway tissue, and they express the SV40 T-antigen (Reddel *et al.*, 1988). Primary cultures of NHBE cells from three individuals were obtained from Clonetics (San Diego, CA). BEAS-2B and NHBE cells were grown on collagen-fibronectin-coated tissue culture plates in serum-free 50% v/v LHC-9 (Biofluids, Rockville, MD)/RPMI 1640 (GIBCO BRL, Gaithersburg, MD) medium supplemented with 100 units/ml each penicillin/streptomycin at 37° and 5% CO_2. Twenty-four hours before transfection, cells were incubated with hydrocortisone-deficient LHC-9/RPMI 1640 medium.

Adenovirus-mediated transfection was utilized with BEAS-2B cells for radioligand binding and electrophoretic mobility shift studies (Forsayeth and Garcia, 1994). BEAS-2B cells were exposed to the transfection mixture (80 μl/10-cm^2 plate adenovirus stock (Forsayeth and Garcia, 1994), 10 μg/10-cm^2 plate pBC12BI-PAFR, 4 μl/10-cm^2 plate DEAE dextran (10 mg/ml), and 4 ml/10-cm^2 plate hydrocortisone-deficient LHC-9 medium) for 2 hr. Transfection was terminated by aspiration of the transfection mixture and washing with a solution of 10% dimethyl sulfoxide for 2 min. This solution was then replaced with hydrocortisone-deficient LHC-9/RPMI 1640 medium. Cells were incubated an additional 48 hr before harvesting for radioligand binding studies. Cells used for electrophoretic mobility shift assay analysis were treated with 100 nM PAF (Bachem, Torrance, CA) for the indicated times 48 hr after transfection. At the time of PAF stimulation, culture medium was replaced with hydrocortisone-deficient LHC-9/RPMI 1640 medium containing 0.25% bovine serum albumin. A PAF stock solution was prepared in the identical medium and then used for cell stimulation.

Transfection by lipofection was utilized for CAT reporter experiments where the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate:L-α-diodeoyl phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) was added to plasmid DNA at a 1:1 w/w ratio (Felgner *et al.*, 1987). Lipid/DNA complexes were allowed to form for 15 min at room temperature before incubating with BEAS-2B cells for 2 hr at 37°. Transient transfection assays were carried out with 5 μg/10-cm^2 plate of the internal control plasmid pCMV-β-galactosidase, 5 μg/10-cm^2 plate pBC12BI-PAFR, and 1 μg/10-cm^2 plate p5xTRE-CAT or as indicated. After incubation the lipid/DNA complexes were removed and replaced with hydrocortisone-deficient LHC-9/RPMI 1640 media. Twenty-four hours after transfection cells were treated with 100 nM PAF, 100 μM WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*]-[1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-yl]-1-(4-morpholinyl)-1-propanone; Boehringer-Ingelheim, Ridgefield, CT), and/or 10 ng/ml TPA (Calbiochem, La Jolla, CA) for CAT reporter experiments. Cells were then incubated an additional 24 hr before harvesting.

Radioligand binding assay. Cell membranes were prepared for BEAS-2B radioligand binding studies. BEAS-2B cells were suspended in cold 50 mM Tris buffer, pH 7.2, and homogenized at 4° with three 15-sec bursts on a Polytron homogenizer at setting 7. Homogenates were centrifuged at 40,000 × *g* for 15 min at 4°, and the resulting pellet was resuspended in 9 volumes of 50 mM Tris buffer, pH 7.2. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL). The binding incubation medium used for BEAS-2B cells was 50 mM Tris buffer containing 5 mM $MgCl_2$, 125 mM choline chloride, and 2.5 mg/ml bovine serum albumin at pH 7.2 (Gomez *et al.*, 1990). Whole cells were utilized for NHBE radioligand binding studies. NHBE cells were resuspended in cold 50 mM Tris buffer, pH 7.2, for protein determination. The binding incubation medium for NHBE cells was 140 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 2 mM $MgCl_2$, 12 mM $NaHCO_3$, 10 mM Tris·HCl, 6.2 mM dextrose and 0.25% bovine serum albumin, pH 7.4 (Herbert, 1992).

For all radioligand binding studies, the assay volume was 1 ml, with a final protein concentration of 80–300 μg of protein/ml for NHBE and BEAS-2B cells. Radioligand binding was performed at 4° for 2 hr. All measurements were made in triplicate in at least three independent experiments. Specific [^{3}H]WEB 2086 (14.1 Ci/mmol; New England Nuclear, Boston, MA) binding was experimentally determined from the difference between radioligand bound in the absence (total) and presence (nonspecific) of 100 μM WEB 2086. Binding reactions were terminated by filtering the incubation medium over glass fiber filters (Whatman GF/B) using a single filter holder apparatus (model FH 224; Hoefer Scientific Instruments, San Francisco, CA). Each filter was rinsed three times with 4 ml of assay buffer. The filters were presoaked in assay buffer for 30 min to decrease nonspecific binding to the filter. Receptor-bound radioactivity retained on the filter was extracted for 16 hr with 9 ml of liquid scintillation fluid (Cytoscint; ICN, Aurora, OH). Radioactivity of each sample was determined by liquid scintillation spectrophotometry.

Detection of PAF receptor transcripts. Poly(A)$^+$ RNA (250 ng) from NHBE cells was transcribed with RT to cDNA using random hexanucleotides (0.2 μg/μl) as primers and the cDNA was then used for PCR. PCR primers used were as follows and numbered from the

translation start site: L1 (5′-GGCTGGGGCCAGGACCCAGA-3′, complementary to nucleotides −104 to −85), H1 (5′-CCTGAGCTCCCCGAGAAGTCA-3′, complementary to nucleotides −165 to −145), C1 reverse primer (5′-CCCGAGCACAAAGATGATGC-3′, complementary to nucleotides +87 to +68) (Mutoh *et al.*, 1993). L1/C1 primers were used for the detection of transcript 1, whereas H1/C1 specific primers were used to test for the presence of transcript 2. PCR reactions contained 200 μM dNTPs, 10% dimethyl sulfoxide, 1× PCR buffer, 1 μM primers, 2.5 units of *Taq* polymerase (Perkin-Elmer, Foster City, CA). Cycling parameters consisted of an initial denaturation at 94° for 4 min, followed by 30 cycles of annealing at 50° for 1 min, extension at 72° for 1 min, and denaturation at 94° for 1 min with a final extension step at 72° for 6 min. PCR products were analyzed by agarose gel electrophoresis on 3% NuSieve GTG (FMC BioProducts, Rockland, ME).

Electrophoretic mobility shift and supershift assays. Mobility shift assays and cellular nuclear protein extraction were performed as described by Camhi *et al.* (1995). DNA binding activity was determined after incubation of 5–10 μg of BEAS-2B nuclear protein extract with 30–60 fmol (20,000–50,000 cpm) of a ^{32}P-labeled 22-mer oligonucleotide encompassing the AP-1 site (5′-CTAGTGATGAGTCAGCCGGATC-3′) (Stratagene) in reaction buffer containing 10 mM HEPES, pH 7.9, 1 mM dithiothreitol, 1 mM EDTA, 80 mM potassium chloride, 1 μg of poly[d(I-C)][d(I-C)], and 4% Ficoll. After a 30 min incubation at 4°, the reaction mixture was electrophoresed on a 4–6% polyacrylamide gel containing 0.5 × Tris-borate-EDTA (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The gel was transferred to 3 mm chromatography paper (Whatman, Maidstone, UK) and dried before exposure to autoradiographic film. Self-competitions were carried out under the same conditions using 10- and 100-fold molar excess of the unlabeled AP-1 oligonucleotide probe. Nonspecific competitions were performed using an unlabeled oligonucleotide probe encompassing a Sp1 transcription factor binding site (5′-GATCGATCGGGGCGGGGCGATC-3′) and a probe encompassing an Oct-1 binding site (5′-GATCGAATGCAAATCACTAGCT-3′) (Stratagene).

For antibody supershift assays, 4 μg of each polyclonal antisera were added subsequent to the oligonucleotide probe and nuclear extract incubation and incubated an additional 60 min at 4°. The polyclonal antibodies against Jun family (sc-044X) and Fos family (sc-253X) proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Fos family antibody is a goat polyclonal IgG antibody with specificity for an epitope corresponding to amino acids 128–152 within a highly conserved domain of human c-Fos and is broadly reactive with human c-Fos and FosB Fra-1 and Fra-2 proteins. The Jun family antibody is a goat polyclonal IgG antibody with specificity for an epitope corresponding to a highly conserved DNA binding domain (residues 247–263) of mouse c-Jun and is broadly reactive with human c-Jun, JunB, and JunD proteins.

CAT assay. Forty-eight hours after transfection by the lipofection technique, CAT and β-galactosidase assays were performed on cell lysates. Protein concentration, as determined using the bicinchoninic acid assay (Pierce), was normalized for transfection efficiency to β-galactosidase expression. CAT activity was determined by the acetylation of [^{14}C]chloramphenicol (55.5 mCi/mmol; New England Nuclear) during a 4-hr incubation at 37°. Substrate and acetylated products were separated by thin-layer chromatography and the percent conversion of [^{14}C]chloramphenicol to the acetylated forms was quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Data analysis. Experimental data for PAF binding studies were analyzed using a nonlinear least-squares regression program, PRISM (GraphPAD Software, San Diego, CA) using a one-site fit model. Experimental values for transcription studies were compared using the one-sample Student's t test. Statistical difference was inferred with $p < 0.05$. The mean values for logarithmically distributed data are reported as the geometric mean value ×/÷ 247 SEM, where SEM is the factor by which the mean is multiplied or divided to obtain a standard error from the mean.

Results

Functional expression of the PAF receptor in human primary bronchial epithelial cells. Saturation isotherms demonstrated specific binding sites for the PAF antagonist [^{3}H]WEB 2086 in human primary bronchial epithelial cells (Fig. 1A). The binding was saturable and best described by interaction of the radioligand with a single population of high affinity binding sites. The equilibrium dissociation constant (K_d) for [^{3}H]WEB 2086 binding was 9.8 nM (range 2.6–36.4 nM; three experiments). The maximal density of binding sites (B_{max}) for [^{3}H]WEB 2086 binding was 42.4 ± 23.3 fmol/mg of protein (three experiments). Nonspecific binding increased linearly with increasing radioligand concentrations in the presence of 100 μM WEB 2086.

The human PAF receptor gene was reported to exist as a single copy gene with two different species of functional mRNA (transcripts 1 and 2) that vary in tissue distribution (Mutoh *et al.*, 1993). We used RT-PCR to examine the expres-

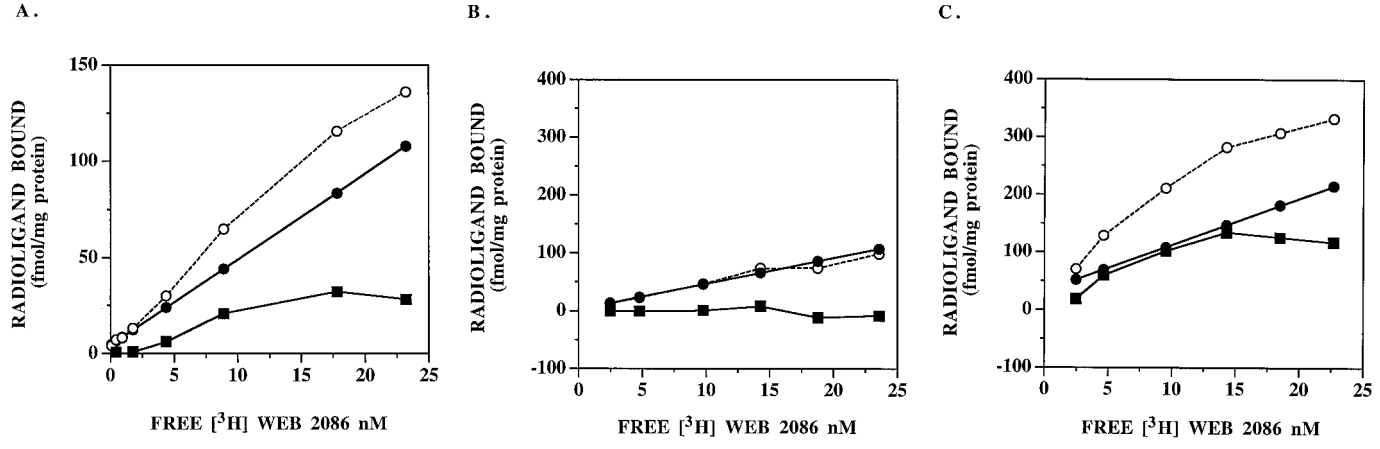

Fig. 1. Saturation isotherms of [^{3}H]WEB 2086 binding. Assay conditions were as described under Materials and Methods. Specific binding (■), best described by a one-site fit, is the difference between binding in the absence (○) and presence of (●) 100 μM WEB 2086. A, Human primary bronchial epithelial cells. Data from one of three experiments are shown. Protein concentration = 290 μg/ml. B, Nontransfected BEAS-2B cells. Data from one of three experiments are shown. Protein concentration = 89 μg/ml. No endogenous PAF receptors were detected in nontransfected BEAS-2B cell membranes. C, Transfected BEAS-2B cells. Data from one of three experiments are shown. Protein concentration = 117 μg/ml.

sion of transcript 1 and/or transcript 2 mRNA in the human primary bronchial epithelial cells. Amplification products by RT-PCR indicated constitutive levels of both transcript 1 (Fig. 2, *lane 2*) and transcript 2 (Fig. 2, *lane 3*) mRNA present in primary cultures of human bronchial epithelial cells. The identity of transcripts 1 and 2 was verified by subcloning and sequencing the RT-PCR products (data not shown).

We examined similarly the bronchial epithelial cell line BEAS-2B for the presence of PAF receptors by radioligand binding. In contrast to the primary bronchial epithelial cells, radioligand binding studies with [^{3}H]WEB 2086 revealed no evidence of specific binding on the BEAS-2B cell line (Fig. 1B).

Expression of the cloned human PAF receptor in BEAS-2B cell line. A full-length genomic clone for the human PAF receptor (Chase *et al.*, 1993) in the expression plasmid pBC12BI-PAFR was transfected into the BEAS-2B cells. At 48 hr post-transfection, BEAS-2B cell membranes displayed specific binding to [^{3}H]WEB 2086 with a dissociation constant of 8.2 nM (range 4.4–15.2; three experiments) (Fig. 1C). Thus, the PAF receptor expressed in transfected BEAS-2B cells had binding properties very similar to those endogenously produced in the human primary bronchial epithelial cells.

Temporal pattern of PAF-induced AP-1 DNA binding activity. Nuclear extracts were prepared from BEAS-2B cells expressing the PAF receptor at the indicated times after stimulation with PAF. The AP-1 DNA binding activity was examined by the electrophoretic mobility shift assay. PAF induced a rapid and sustained increased level of AP-1 DNA binding activity, observed within 15 min, and sustained for up to 4 hr (Fig. 3A, *lanes 1–6)*. The specificity of AP-1 binding activity was demonstrated by the ability of unlabeled AP-1 oligonucleotide to compete with the radiolabeled AP-1 sequence for binding of nuclear factors (Fig. 3B, *lanes 2* and *3).* Three protein/DNA bands were induced by PAF and all three bands were specific for AP-1. Two oligonucleotide probes containing an unrelated Sp1 consensus sequence or Oct-1 consensus sequence showed no inhibition of binding of the radiolabeled AP-1 probe (Fig. 3B, *lanes 4–7)*.

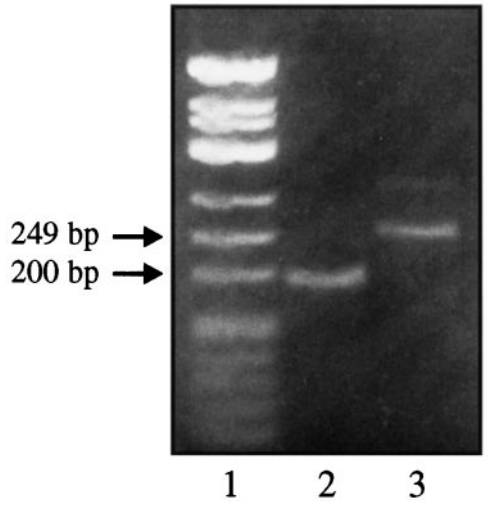

Fig. 2. RT-PCR analysis for detection of transcripts 1 and 2 in human primary bronchial epithelial cells. mRNA isolated from human primary bronchial epithelial cells was transcribed into cDNA using reverse transcriptase and random hexanucleotides as primers. The cDNA was used for PCR analysis as described in Materials and Methods. Amplification products from one of three experiments are shown using the primer pairs L1/C1 (191-bp fragment) and H1/C1 (252-bp fragment). C1 is complementary to the genomic sequences between +68 and +87 to the ATG. Primer L1 is the sequence between −104 and −85 in transcript 1. Primer H1 is the sequence between −165 and −145 in the transcript 2. *Lane 1*, molecular weight marker with 200- and 249-bp indicated; *lane 2*, RT-PCR product representing transcript 1; *lane 3*, RT-PCR product representing transcript 2.

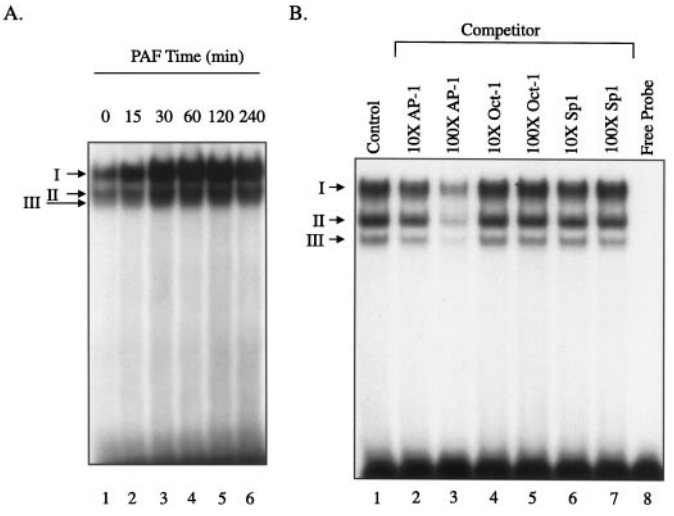

Fig. 3. Electrophoretic mobility shift assay showing time-dependent stimulation and specificity of PAF-induced AP-1 binding activity in BEAS-2B cells transfected with the PAF receptor. Nuclear extracts were prepared from transfected BEAS-2B cells stimulated with 100 nM PAF. Equal quantities of nuclear protein (5 μg) were analyzed for AP-1 binding activity by electrophoretic mobility shift assay using 30 fmol of an oligonucleotide containing a consensus TRE sequence (AP-1 site). *Arrows*, AP-1 specific complexes. A, BEAS-2B cells were stimulated at the time points indicated (*lanes 1–6*). B, Competition of AP-1 binding was carried out with 10- and 100-fold molar excess of the unlabeled AP-1, Oct-1, and Sp1 probes using nuclear extracts from cells stimulated with PAF for 60 min (*lanes 2–7*).

Characterization of the AP-1 transcription factor complex. Gel supershift assays were performed to determine whether AP-1 components, i.e., Fos and Jun were present in the protein complexes binding to the AP-1 consensus sequence. With nuclear extracts from PAF-stimulated BEAS-2B cells transfected with the PAF receptor, antibodies to the Fos and Jun family proteins induced supershifts of the DNA-protein complex I (Fig. 4, *lanes 3–5)*. The supershift observed with the Fos antibody is consistent with this antibody binding to the AP-1 DNA/Fos/Jun complex and decreasing its mobility. By contrast, the Jun antiserum primarily inhibits DNA binding, which is consistent with the specificity of the antibody to the highly conserved DNA binding domain of c-Jun. An antibody to a nonrelated protein, NF-p50, did not induce a supershift of the DNA-protein complexes (Fig. 4, *lane 6)*. In an attempt to identify proteins in the AP-1 complex II and III, supershifts using an antibody specific for CREB-binding protein and p300 were performed. Antibodies to CREB-binding protein/p300 did not induce a supershift of AP-1 complexes (data not shown).

Induction of AP-1 mediated transcription through the PAF receptor. The ability of PAF to activate AP-1-mediated transcription was investigated. BEAS-2B cells cotransfected with the human PAF receptor expression plasmid pBC12BI-PAFR and the AP-1 reporter plasmid p5xTRE-CAT were stimulated with 100 nM PAF or 10 ng/ml TPA and subsequently analyzed for CAT activity. The results of five

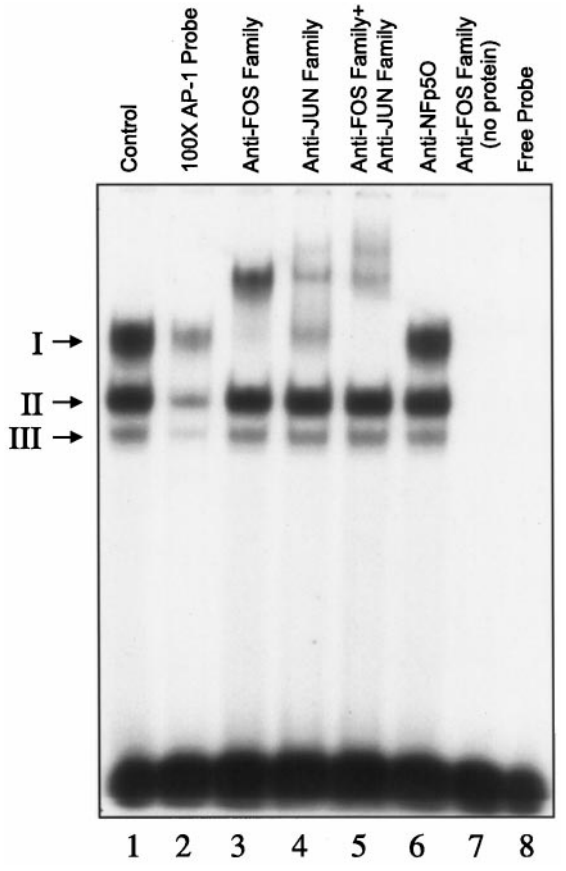

Fig. 4. Identification of Fos and Jun in the DNA-protein complexes induced by PAF. Nuclear extracts (10 μg) from transfected BEAS-2B cells stimulated with PAF (100 nM for 1 hr) were incubated in the presence of specific antibodies (4 μg/sample) against Fos family and Jun family proteins. Samples were analyzed by electrophoretic mobility shift assay (4% gel) using 60 fmol of an oligonucleotide probe containing the consensus AP-1 sequence. *Control*, PAF receptor transfected BEAS-2B cells stimulated with PAF for 1 hr (*lane 1*). Competition of AP-1 binding was carried out with 100-fold molar excess of the unlabeled AP-1 probe (*lane 2*). The anti-Fos family and anti-Jun family antibodies induced shifts of the DNA-protein complex I specific for AP-1 (*lanes 3–5*). *Arrows*, AP-1 specific complexes.

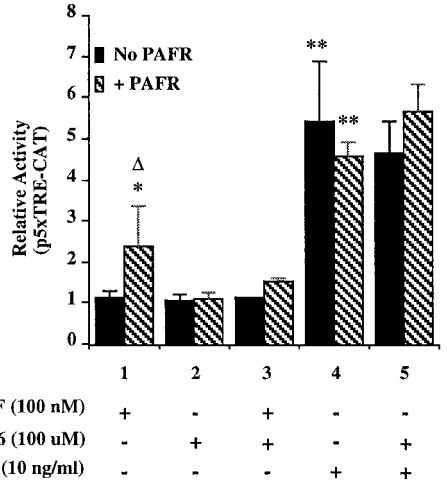

Fig. 5. PAF induction of AP-1-mediated transcription in BEAS-2B cells. CAT activity was determined following cotransfection of p5xTRE-CAT reporter plasmid and the β-galactosidase expression plasmid pCMV-β-galactosidase into BEAS-2B cells as described in Material and Methods. The plasmid pBC12BI-PAF receptor (*PAFR*) was included in transfections as indicated. Cells were stimulated with PAF (100 nM), WEB 2086 (100 μM), and/or TPA (10 ng/ml) for 24 hr then harvested for CAT and β-galactosidase determination. The transcriptional activity of the AP-1 reporter plasmid was calculated relative to the level of CAT activity for untreated BEAS-2B cells (activity = 1). The activity of AP-1 is expressed as the geometric mean *x*/ 247 SEM for five experiments. All measurements were performed in duplicate. *, $p < 0.03$, and **, $p < 0.005$, significant difference from basal activity (activity = 1) as determined by Student's t test. Δ, $p < 0.05$, significant difference from condition 1 without the PAF receptor present as determined by the Student's t test.

experiments shown in Fig. 5 demonstrate that TPA induced significant increases in CAT activity over that in unstimulated cells independent of the expression of the PAF receptor ($p < 0.005$; Fig. 5). In comparison, PAF significantly induced AP-1-mediated transcription only in BEAS-2B cells expressing the PAF receptor ($p < 0.03$; Fig. 5). The PAF antagonist WEB 2086 blocked PAF-induced AP-1 transcriptional activity (Fig. 5, *condition 3)* but not the TPA effect (Fig. 5, *condition 5)*, indicating the specificity of the PAF response. The control expression plasmid pBC12BI, without the PAF receptor cDNA did not respond to PAF (data not shown). Results indicate that the transfected BEAS-2B cells appeared to have intact signaling properties capable of enhancing AP-1 activity and that PAF acting via the PAF receptor induced AP-1 transcriptional activity.

Discussion

The results of our study demonstrate that PAF receptors are expressed on the surface of primary human bronchial epithelial cells and that PAF, acting through its receptor, is capable of inducing AP-1 DNA binding activity and AP-1-directed transcription. This expression and function of the PAF receptor provides evidence for an involvement of bronchial epithelial cells in PAF-induced inflammatory responses of the lung.

Specific PAF-receptor binding sites have been identified previously on human platelets, polymorphonuclear leukocytes, eosinophils, monocytes, macrophages, and human lung tissues (Venable *et al.*, 1993). We have demonstrated specific binding sites for the PAF antagonist [^{3}H]WEB 2086 on primary human bronchial epithelial cells with an affinity (K_d) of 9.8 nM and a binding capacity (B_{max}) of 42.3 fmol/mg of protein (equivalent to 6.1 fmol/10^5 cells). The K_d for binding of [^{3}H]WEB 2086 to the PAF receptor on primary bronchial epithelial cells was comparable to the dissociation constant (K_d = 8.2 nM) for the human cloned PAF receptor transiently expressed in BEAS-2B cells. We and others have found similar K_d values for binding of [^{3}H]WEB 2086 to the cloned PAF receptor when expressed in COS-7 and HL-60 cells (Ye *et al.*, 1991; Honda *et al.*, 1991; LeVan *et al.*, 1997). The number of specific binding sites for [^{3}H]WEB 2086 on human primary bronchial epithelial cells was comparable to that found on human nasal epithelial cells (2.1 fmol/10^5 cells) and human lung (140 fmol/mg of protein) (Hwang *et al.*, 1985; Kang *et al.*, 1994). However, it was much greater than that found on guinea pig tracheal epithelial cells (0.172 fmol/10^5 cells) (Herbert, 1992).

We propose that airway inflammation may involve (in part) a response of bronchial epithelial cells to PAF via its receptor resulting in the *trans*-activation of bronchial epithelial cell target genes that possess and are activated by functional AP-1 sequences. Results of our transient transfection assays using the bronchial epithelial cell line BEAS-2B re-

vealed that PAF can activate transcription from AP-1 transcriptional elements and that this activity was initiated by the PAF receptor-ligand binding. The specificity of the response was demonstrated by the inhibition of transcriptional activation by the PAF antagonist WEB 2086. In addition, PAF was capable of inducing AP-1 DNA binding activity within 15 min in transiently transfected BEAS-2B cells as shown by electrophoretic mobility shift assays. This is an unusually early response for AP-1 DNA binding activity and is most likely due to stimulation of preexisting components rather than the synthesis of new Fos and Jun proteins. Our results suggest that this pathway of AP-1 activation occurs in addition to that involving increased mRNA levels for AP-1 components reported by others. These previous studies in other cell types demonstrated that PAF, via binding to the PAF receptor, promoted induction, i.e., within 30 min of c-*fos* mRNA in human B-lymphoblastoid cells, human A431 epidermoid carcinoma cells, human neuroblastoma cells and in the hippocampus (Schulam *et al.*, 1991; Marcheselli and Bazan, 1994; Tripathi *et al.*, 1992; Squinto *et al.*, 1989). Also *c-jun* mRNA was induced through interaction of PAF with its receptor in human lung fibroblasts, human neuroblastoma cells, and a human B cell line (Squinto *et al.*, 1989; Schulam *et al.*, 1991). In this study, three specific AP-1 DNA binding complexes were observed. Our studies show both Fos and Jun proteins present in complex I. These proteins have been found previously to bind to AP-1 consensus sequence either as Jun/Jun or Jun/Fos dimers (Angel and Karin, 1991). Because anti-Fos antibodies induced almost a complete shift of complex I, this would indicate the prevalence of Fos/Jun heterodimers in this complex with Jun/Jun homodimers possibly as a minor component.

That PAF is capable of induction of the AP-1 signaling pathway in bronchial epithelial cells may have several implications. First, by activating AP-1 mediated transcription, PAF may participate in the regulation of target gene expression under physiological and pathological conditions. Second, sequence analysis of the regulatory regions for transcript 1 and transcript 2 revealed that the putative promoter region for the PAF receptor has consensus sequences for the transcription factors AP-1, AP-2, Sp-1, and NF-κB. Thus PAF may autoregulate its own receptor expression via AP-1. Because these transcripts were shown to have different tissue distributions, the PAF receptor may be differentially regulated in different cell types.

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