

Inhibition by Platelet-activating Factor of Src- and Hepatocyte Growth Factor-dependent Invasiveness of Intestinal and Kidney Epithelial Cells

PHOSPHATIDYLINOSITOL 3'-KINASE IS A CRITICAL MEDIATOR OF TUMOR INVASION*

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This study was designed to characterize platelet-activating factor receptor (PAF-R) expression and function in normal and cancerous human colonic epithelial cells. PAF-R gene transcripts were analyzed by reverse transcription-polymerase chain reaction and Southern blot, using three sets of primers corresponding either to the coding region of the human PAF-R sequence (polymerase chain reaction product: 682 base pairs (bp)) or to the leukocyte- and tissue-type transcripts of 166 and 252 bp, respectively. An elongated splice variant was identified in the 5'-untranslated region of the tissue-type PAF-R transcript (334 bp) in colonic epithelial crypts and tumors. In human colonic PCmsrc cells transformed by *c-src* oncogene, the hepatocyte growth factor (HGF)-dependent invasiveness of collagen gels was abolished by 0.1 μ M PAF and restored by the PAF-R antagonists WEB2086 and SR27417. PAF blocked HGF-induced tyrosine phosphorylation of p125 focal adhesion kinase. The phosphatidylinositol 3'-kinase (PI3'-K) inhibitors wortmannin and LY294002 totally blocked the HGF-induced invasion. Similar effects were observed in *ts-srcMDCK* kidney epithelial cells transformed by a *v-Src* temperature-sensitive mutant: (i) PAF and wortmannin exerted additive inhibitory effects on Src-induced invasion and (ii) activated and dominant negative forms of p110 α PI3'-K, respectively, amplified and abrogated the Src- and HGF-dependent invasiveness of parental and *ts-srcMDCK* cells. We also provided the first evidence for the contribution of rapamycin-insensitive, pertussis toxin-dependent G-protein pathways to the integration of the signals emerging from activated Met and PAF receptors. These results indicate that PI3'-K is a critical transducer of invasiveness and strongly suggest that PAF exerts a negative control on invasion by inhibiting this signaling pathway. A possible beneficial role of PAF analogs on tumor invasion is therefore proposed.

Platelet-activating factor (PAF),¹ 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, is implicated in human inflammatory processes and gastric ulceration disorders (1). In the intestine, PAF contributes to diarrhea in ulcerative colitis (2). It is a potent lipid mediator produced by neutrophils, eosinophils, monocytes, macrophages, platelets, and endothelial cells via two main routes of biosynthesis: the remodeling pathway and a second pathway termed *de novo*. Products of phospholipid catabolism (PAF and eicosanoids) are detected in the gastrointestinal mucosa and epithelial cells during the inflammatory process and neoplastic progression (3, 4). Colonic epithelium has been reported to synthesize and secrete PAF acetylhydrolases, which constitute a major pathway for PAF degradation (5). Increased levels of PAF in intestinal mucosa have been reported in experimental models of colitis, Crohn's disease, and ulcerative colitis (2, 6, 7). Numerous clinical and experimental observations reveal that colonic cancer is increasingly frequent after inflammatory bowel disease, ulcerative colitis, primary sclerosing cholangitis, and Crohn's disease (3, 8, 9), suggesting that PAF and its receptors initiate the cancerous progression through inflammatory disorders in the gastrointestinal mucosa. On the other hand, anti-inflammatory drugs are associated with a lower risk of colon cancer and reduced mortality due to this disease (10).

In view of the information above, we are now asking whether functional PAF receptors (PAF-R) are present in both normal and transformed human colonic epithelial cells. As a first step toward answering this question, we investigated 1) the expression of PAF-R transcripts by reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot in normal and transformed human colonic epithelial cells, 2) the effects of PAF on invasion by tumor cells, using two models of PCmsrc human colonic epithelial cells and *ts-srcMDCK* canine kidney epithelial cells transformed by the *src* oncogene (11, 12), and 3) the functional relationships between PAF receptors and the signaling pathways involved in cell adhesion and invasion, namely p125^{FAK} focal adhesion kinase, phosphatidylinositol 3'-kinase (PI3'-K), and the E-cadherin/catenin invasion suppressor system connected to the actin cytoskeleton via α -catenin (13).

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¹ The abbreviations used are: PAF, platelet-activating factor; PAF-R, platelet-activating factor receptor; HGF, hepatocyte growth factor; PI3'-K, phosphatidylinositol 3'-kinase; FAK, focal adhesion kinase; PTx, pertussis toxin; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); TBS, Tris-buffered saline; PBS, phosphate-buffered saline; UTR, untranslated region; mAb, monoclonal antibody; MDCK, Madin-Darby canine kidney; PMSF, phenylmethylsulfonyl fluoride.

In the present investigation, we showed the following. (i) Specific tissue-type PAF-R transcripts are clearly identifiable in human intestinal epithelial cells at various stages of cancerous progression and cell differentiation; (ii) upon PAF addition, there was complete reversion of the Src- and HGF-dependent invasiveness of intestinal and kidney epithelial cells; (iii) the PI3'-K inhibitors wortmannin and LY294002 mimic and cooperate with PAF against invasion; (iv) there was phosphorylation of p125^{FAK} by HGF that was completely blocked by PAF; (v) constitutively activated PI3'-K and dominant negative forms of the PI3'-K p110 α catalytic subunit respectively induced and abrogated the invasiveness of parental and Src-transformed MDCK cells; and (vi) the HGF- and PAF-dependent invasion pathways were sensitive to pertussis toxin (PTx), suggesting that heterotrimeric G protein components (G α β γ dimers) are involved in the signaling cascades that affect the invasiveness of Src- and Met-transformed epithelial cells.

A preliminary report of the results presented here has been published in abstract form (14).

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium was from Life Technologies, Inc., and fetal calf serum from Eurobio. [γ -³²P]ATP was from ICN; the enhanced chemiluminescence (ECL) immunodetection system and Protein G-Sepharose were from Amersham Pharmacia Biotech; PAF and wortmannin were from Calbiochem. Fatty acid-free bovine serum albumin, Geneticin, PTx, rapamycin, phenylmethylsulfonyl fluoride (PMSF), Triton X-100, Nonidet P-40, and LY294002 were purchased from Sigma. SR27417A and WEB2086 were, respectively, obtained from Sanofi and Boehringer Ingelheim (France). The anti-phosphotyrosine monoclonal antibody (mAb) 4G10, and the anti-p125^{FAK} mAb (clone 77) were from Upstate Biotechnology, Inc. (UBI) and Transduction Laboratories, respectively.

Cell Lines, Cell Transfection, and Tissue Samples—The human intestinal epithelial cell lines Caco-2, PC/AA C1, CFI-3, HT-29, and HT29-MTX were routinely grown in 6-cm diameter Petri dishes, as described previously (15–17). CFI-3 cells were established from the intestinal epithelia of a fetus with cystic fibrosis. This cell line does not produce any tumor in athymic nude mice and is relatively undifferentiated (15). The PC/AA/C1 cell line was established from a colonic adenoma in a patient with familial adenomatous polyposis. This cell line is nontumorigenic and exhibits a mucinous phenotype (16). After transfer of the activated *c-src* oncogene, PCmsrc cells became susceptible to invasiveness upon addition of HGF (11). The colonic cancer cell lines HT-29, Caco-2, and HT29-MTX cells, respectively, exhibit undifferentiated, enterocyte-type, and mucinous-type phenotypes (17). Human colonic crypts were obtained from fresh samples, as described previously (15).

MDCK canine kidney epithelial cells, transformed by a temperature-sensitive mutant of v-Src (ts-srcMDCK cells, Cl2), exhibit an invasive phenotype at the permissive temperature of 35 °C, but are not invasive at the restrictive temperature of 40 °C for v-Src activity (12).

Transformed ts-srcMDCK cells were stably transfected by the dominant-negative mutant of bovine PI3'-K p110-EcoS. This truncated form of PI3'-K p110 α contains the binding site for the regulatory p85 subunit but lacks the catalytic domain (18). Cells were transfected by the LipofectAMINETM method, using 25 μ l of reagent/well and 10 μ g of plasmid for 2 $\times$ 10⁶ cells (Life Technologies, Inc.). After 48 h, cells were selected over a period of 2 weeks with 500 μ g/ml G418. Individual resistant colonies were then isolated, picked, and expanded, and the expression of the mutant PI3'-K p110-EcoS form was assessed by Northern blot. Parental MDCK cells and MDCKp110* cells, stably transfected by the activated form of bovine p110* α after addition of the C-terminal farnesylation signal from Ha-Ras, were a generous gift from Dr. J. Downward (19).

Normal human leukocytes were isolated from peripheral blood and purified by leukapheresis and countercurrent centrifugation elutriation (20). Human leukemic HL-60 promyelocytes were cultured as described previously (20). Specimens from patients who had undergone surgery for colonic cancer were obtained from the Center de Chirurgie Digestive (Prof. R. Parc, Hôpital Saint-Antoine, Paris, France). Tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until use. Individual colon carcinomas were staged according to Dukes' classification, as modified by Astler and Collier (17).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and

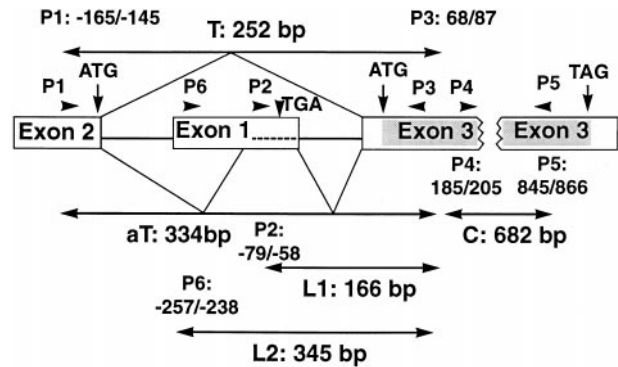


FIG. 1. Schematic representation of the PCR and Southern analysis of PAF-R expression in normal and cancerous human intestinal epithelial cells and leukocytes. The primers and corresponding amplification product corresponding to each target transcript were as follows: (i) coding region C, primers P4 (sense in exon 3) (5'-CGGACATGCTCTTCTTGATCA-3') and P5 (antisense) (5'-GTC-TAAGACACAGTTGGTGCTA-3') (682 bp); (ii) antisense primer P3, common to the leukocyte- and tissue-type transcripts (5'-CCCGAGCA-CAAAGATGATGC-3'); (iii) the sense primers P2 in exon 1, specific for the leukocyte-type transcript L (166 bp; 5'-ACACACGGTCACTGC-AGCTGAA-3') or P1 in exon 2, specific for the tissue-type transcript T (334 bp; 5'-CCTGAGCTCCCCGAGAAGTCA-3'); (iv) the sense primer P6 in exon 1, upstream primer P2, corresponding to the leukocyte-type transcript and a PCR product of 345 bp (5'-TCTGTGCAGTGCCAG-GTCT-3'). aT, additional tissue-type transcript: 334 bp (dashed line in exon 1).

Southern Blot—Total RNA was isolated by guanidinium isothiocyanate extraction and cesium chloride density gradient ultracentrifugation (11). To eliminate contamination by genomic DNA for RT-PCR analysis, RNA samples were treated with RNase-free DNase. First-strand cDNA was synthesized from 5 μ g of total RNA, using 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), and random hexanucleotides (5 μ M). A fraction of the corresponding cDNA (1/10) was then amplified in 20 μ l of reaction buffer containing 200 μ M amounts of each dNTP, 0.5 μ M amounts of each primer, and 1 unit of AmpliTaq polymerase (Perkin-Elmer). Samples displaying a clear PCR product of glyceraldehyde-3-phosphate dehydrogenase were considered useful. Negative control reactions in which no RT was performed were included in our experiments, to avoid artifacts due to genomic DNA contamination.

As shown in Fig. 1, four sets of primers were designed to amplify the RT-PCR products of the PAF-R gene transcripts corresponding to the coding region, and the tissue- and leukocyte-type 5'-UTR sequences (21–23). Thirty amplification cycles, each comprising 30 s at 94 °C, 1 min 30 s at 60 °C, and 1 min 30 s at 72 °C, were run in an automatic thermal Robocycler Gradient 96 (Stratagene). The reaction was initiated by a 5-min incubation at 94 °C and ended by a 7-min final extension at 72 °C. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Southern blot analysis was performed to verify the authenticity of the PCR products, using two internal probes: probe 1 (5'-CATCAAGACTGCTCAGGCCAACA-3') and probe 2 (5'-ACCAGGACCAGCTGATCATTC-3').

Subcloning and Sequencing of PCR Fragments—To subclone the RT-PCR fragments corresponding to the 5'-UTR of the tissue- or leukocyte-type PAF-R transcript, amplicons were eluted from the agarose gels using the Qiagen kit (Coger, France) and cloned in a PCR-TRAP vector (GenHunter Corp.). Sequencing was performed using the Lseq and Rseq primers and the Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.).

Northern Blot Analysis—Northern blot analysis for the expression of PAF-R and PI3'-K in MDCK cells was performed as described previously (11). RNA samples underwent electrophoresis in 1% agarose, 2.2 mol/liter formaldehyde gels, were transferred to Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech, United Kingdom), and hybridized with the ³²P-labeled probes (Megaprime, Amersham Pharmacia Biotech). The relative amount of RNA in each lane was judged to be similar by comparing the ethidium bromide or methylene blue staining of the ribosomal bands. The specific probes used were human PAF-R cDNA (21) and the EcoRI restriction fragment of the p110 α cDNA corresponding to the N-terminal fragment of the dominant negative mutant of the enzyme (18).

Invasion of Type I Collagen Gels—Petri dishes were filled with 1.2 ml

of neutralized type I collagen (UBI) and incubated overnight at 37 °C to allow gelification. Cells were harvested using Moscona buffer and trypsin/EDTA, and seeded on top of the collagen gels. Cultures were incubated at 37 °C for 24 or 48 h in the presence of PAF, alone or combined with one of its receptor antagonists WEB 2086 and SR27417A, or with one of the PI3'-K inhibitors wortmannin and LY294002. The invasiveness of PCMssrc and ts-srcMDCK cells was measured by the depth of cell migration inside the gel, using an inverted microscope controlled by a computer program (24). Invasive and superficial cells were counted in 12 fields of 0.157 mm². The invasion index was expressed as the percentage of cells invading the gel over the total number of cells.

Immunoprecipitation and Western Blot of p125^{FAK}—Subconfluent PCMssrc cells in 6-cm diameter Petri dishes were starved for 24 h and then incubated for 24 h in the presence of 10 units/ml HGF, alone or combined with 10⁻⁷ M PAF, or without either HGF or PAF (control). Control and treated PCMssrc cells were then rinsed twice with PBS containing 0.1 mM sodium orthovanadate, and lysed at 100 °C in 0.5 ml of 1% SDS, and 10 mM Tris-HCl (pH 7.4). Cell lysates (1–2 mg of protein) were boiled for an additional 5 min, and centrifuged for 5 min at 14,000 × g. The supernatants were then adjusted for protein content. Total lysate (100 µl) containing 600–800 µg of protein was diluted in 400 µl of H₂O and 500 µl of 2× immunoprecipitation buffer (1× buffer: 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM sodium orthovanadate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 0.5% Nonidet P-40, 40 mM β-glycerophosphate, and 10 µg/ml each of the protease inhibitors leupeptin and aprotinin). Next, these samples were subjected to immunoprecipitation for a 1-h incubation in the presence of the anti-p125^{FAK} mAb clone 77 directed against the kinase domain of FAK (Transduction Laboratories). This step was followed by another hour of incubation with Protein G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). All incubations were carried out with mixing at 4 °C. Immunoprecipitates were washed four times in 1× immunoprecipitation buffer and denatured by boiling in SDS sample buffer.

For the Western blots, total proteins or immunoprecipitates were submitted to electrophoresis in 7.5% polyacrylamide gel and blotted on nitrocellulose membranes (Hybond-C extra, Amersham Pharmacia Biotech). Membranes were rinsed twice in TBS for 5 min, the nitrocellulose was blocked overnight in TBS plus 5% bovine serum albumin (Fraction V, Sigma), and probed for 1 h with anti-phosphotyrosine mAb 4G10 (UBI). The blots were then washed in TBS containing 0.1% Tween 20, probed for 1 h with horseradish peroxidase-conjugated monkey anti-mouse IgG, and revealed by the ECL Western detection system (ECL, Amersham Pharmacia Biotech). To determine the relative amounts of FAK protein, the same nitrocellulose membranes were stripped and incubated with the anti-FAK mAb.

Tyrosine Phosphorylation of β-Catenin—ts-srcMDCK cells in culture were washed three times with phosphate-free RPMI 1640 containing 2% dialyzed fetal calf serum (Life Technologies, Inc.) and labeled for 2 h with 0.5 mCi/ml carrier-free [³²P]orthophosphate (Amersham Pharmacia Biotech, Gent, Belgium). Cells were then detached with an E-cadherin-saving procedure, and incubated in suspension for 30 min at the nonpermissive temperature of 40 °C in the presence of HGF, either alone or combined with PAF, and were further aggregated at 40 °C for 30 min. Control and treated ts-srcMDCK cells were lysed in PBS containing 1% Nonidet P-40 and 1% Triton X-100 and the following phosphatase or protease inhibitors (all from Sigma): PMSF (1.7 mM), leupeptin (21 µM), aprotinin (10 µg/ml), sodium pyrophosphate (10 mM), sodium fluoride (10 mM), and sodium vanadate (1 mM). Equal amounts of trichloroacetic acid-precipitable material were incubated for 3 h at 4 °C with the anti-phosphotyrosine mouse mAb PY20 (ICN Immunobiologicals, Costa Mesa, CA), and then incubated for 1 h with Protein G-Sepharose 4 fast flow beads. The beads were washed three times with lysis buffer, and tyrosine-phosphorylated proteins were eluted three times for 15 min with the same buffer containing 1 mM phospho-L-tyrosine and 10 mM phenylphosphate. Eluted molecules were then immunoprecipitated for 2 h at 4 °C with 20 µl of the β-catenin anti-serum 1522, and for 1 h with Protein G-Sepharose 4 fast flow beads. The immune serum 1522 was raised against the synthetic peptide PGD-SNQLAWFDTDL corresponding to the C-terminal part of mouse β-catenin, and recognized a 95-kDa band on Western blots (25).

PI3'-K Assay—Immunoprecipitation and PI3'-K assay were carried out as described previously (26–28). The ts-srcMDCK cells (5 million cells/dish) were cultured for 24 h in the absence of fetal calf serum and then incubated for 18 h at 37 °C in the presence of HGF (10 units/ml) alone or combined with 0.1 µM PAF. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of Nonidet P-40 lysis buffer (10 mM Tris, pH 7.4, containing 0.15 M NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 1 mM PMSF,

and 5 µg/ml each aprotinin, pepstatin, and leupeptin). Lysates were centrifuged at 12,000 × g for 10 min at 4 °C. Cell extracts containing 1 mg of protein were incubated for 2 h at 4 °C with 4 µg of the anti-phosphotyrosine mAb 4G10 and 40 µl of Protein G-Sepharose, and the immune complexes were washed twice with lysis buffer, twice with PBS supplemented with 1 mM sodium orthovanadate, twice with 0.5 M LiCl in 10 mM Tris-HCl (pH 7.4), and twice with kinase buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, and 5 mM MgCl₂). The beads were resuspended in 50 µl of kinase buffer containing 10 µM ATP, 20 µCi of [γ-³²P]-ATP, and 0.4 mg/ml of a sonicated mixture of phosphatidylserine and phosphatidylinositol (1:1, w/w). The tubes were incubated at 30 °C for 15 min, and the reaction was terminated by addition of 100 µl of 1 M HCl. Phospholipids were extracted with 350 µl of a 1:1 mixture of chloroform/methanol, and the organic phase was washed three times with methanol, 1 M HCl (1:1, v/v). The lipids were desiccated, and the pellets were redissolved in 20 µl of chloroform/methanol, 1 M HCl (200:100:1) and chromatographed on thin layer chromatography plates (precoated with potassium oxalate and backed at 120 °C for 1 h just before use) in chloroform, methanol, 4 M NH₄OH (9:7:2, v/v).

RESULTS

Detection of PAF-R Gene Transcripts in Human Colonic Cell Lines and Tumors—The human PAF-R is encoded by a unique gene composed of two 5'-noncoding exons, each driven by distinct promoters (23). The transcripts originating from exons 1 and 2 are alternatively spliced to a common acceptor site on the third exon encoding the functional PAF-R protein. Transcripts 1 and 2 have both been detected in heart, lung, spleen, and kidney, whereas transcript 1 mainly accumulates in peripheral leukocytes.

Using the pair of primers P4/P5 encompassing the 682-bp internal region of the human PAF-R mRNA coding sequence (amino acids 63–289), 682-bp amplicons of the expected size were clearly identified in freshly isolated normal colonic epithelial crypts and cultured human colonic cancer cell lines (data not shown). PAF-R gene expression was detected in human colon cancer resections (Dukes' stages B2 and C1) and their distant nontransformed colonic mucosa. As positive control, PAF-R expression was characterized in normal and leukemic human leukocytes.

We next designated two sets of primers corresponding to either leukocyte-type 1 transcripts (P2/P3 primers) or tissue-type 2 transcripts (P1/P3 primers), in order to examine the expression of both transcripts in colonic epithelial cells. Leukocyte-type transcripts (166 bp) were weakly expressed in both normal and transformed intestinal epithelial cells (Fig. 2, A and B). In contrast, the tissue-type transcript was strongly expressed in intestinal cells, as a PCR product of the expected size (252 bp), as compared with its expression in leukocytes (Fig. 2C). This 252-bp product was revealed with an additional 334-bp variant (aT). After cloning and sequencing, we demonstrated that the tissue-type 252-bp sequence is identical to the PAF-R transcript 2 as reported previously (22). On the other hand, we observed that the additional tissue-type 334-bp product (aT) sequenced in Caco-2 cells was lengthened by the following 82 nucleotides at the 3'-end of exon 1, as shown in Fig. 1: ACAGCATAGAGGCTGAGGCTGGGGCCAGGACCCAGACAGAGACACACGGTCACTGCAGCTGAAGCCGCTGCCCTGCTACAG (dashed line in exon 1 of Fig. 1).

This fragment was produced by alternative splicing, because the apparent consensus splice donor and acceptor sites GT/AG were identified at the boundaries of this additional aT sequence. The presence of this splice variant in the 5'-untranslated region of the PAF-R transcript may be associated with differential regulation and translational efficiency of PAF-R mRNA in normal and transformed intestinal cells. Because the splice variant of the tissue-type transcript may serve as a template for amplification of the 166-bp product, we used the P6 primer upstream of the splice site in exon 1 to confirm the expression of the leukocyte-type transcript in intestinal epithelial

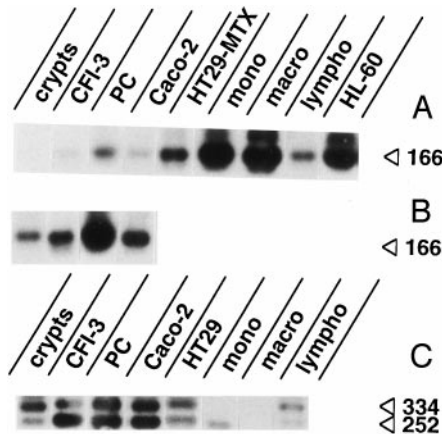


FIG. 2. Expression of the leukocyte- and tissue-type PAF receptor transcripts in 5'-UTRs by RT-PCR and Southern blot analysis in human intestinal epithelial cells and leukocytes. The following cell types were tested: Freshly isolated human colonic crypts (crypts), SV40LT-immortalized CFI-3 intestinal epithelial cells, the human colonic epithelial cancer cell lines Caco-2 (enterocyte-like), HT29 (mainly undifferentiated), and HT29-MTX (mucin-secreting), peripheral human monocytes (*mono*) and their derived macrophages (*macro*), lymphocytes (*lympho*), and human promyelocytic leukemia cells HL-60. Films corresponding to the leukocyte-type transcripts (166 bp) were exposed for 90 min (A) or 6 h (B); those corresponding to the tissue-type transcripts (252 and 334 bp in C) were exposed for 1 h.

lial cells HT-29, Caco-2, and PCmsrc (data not shown).

Effects of HGF, PAF, and PI3'-K on Invasion and Tyrosine Phosphorylation of p125^{FAK} in Colonic and Kidney Epithelial Cells Transformed by the *src* Oncogene—Cell-cell and cell-matrix adhesions play an important role in determining the structural organization of polarized epithelial cells and the behavior of cancer cells toward migration, invasiveness, and metastasis. One major target of activated PAF-R is the cytoskeleton, which is involved in controlling of cell shape, chemotaxis and migration (29, 30). Focal adhesion kinase (p125^{FAK}) is a major point of convergence for the actions of a variety of effectors that affect cell morphology, adhesion, and locomotion, including Src, PI3'-K, and the HGF receptor Met (31). We therefore investigated the effects of PAF on the Src- and HGF-dependent invasiveness of transformed intestinal and kidney epithelial cells (11, 12), in relation with the status of PI3'-K and p125^{FAK}.

As shown in Fig. 3, 0.1 μ M PAF completely blocked the HGF-dependent invasiveness of human colonic epithelial PCmsrc cells in collagen gels. Half-maximal inhibition occurred at 10 nM PAF. The PAF-R antagonist 10 nM reversed, by 100%, the inhibition caused by 0.1 μ M PAF. At 1 nM, another PAF-R antagonist, SR27417A, inhibited by 50% the response to PAF, and total inhibition was observed at 10 nM SR27417A. When both antagonists were tested at 10 nM, they failed to induce invasiveness, suggesting that the behavior of the HGF-stimulated PCmsrc cells was not affected by endogenous PAF. In PCmsrc cells, the action of PAF was mimicked by 10 nM wortmannin (Fig. 3) and LY294002, another structurally unrelated PI3'-K inhibitor. At 10 μ M, LY294002 completely blocked HGF-stimulated invasiveness.

As an extension of these studies, we observed that HGF induced a marked rise in the level of tyrosine phosphorylation of p125^{FAK} in PCmsrc cells (Fig. 4). The anti-p125^{FAK} antibody immunoprecipitated similar amounts of FAK protein in control and HGF/PAF-treated cells. PAF alone failed to induce any detectable change in tyrosine phosphorylation, but completely blocked the HGF-induced phosphorylation of p125^{FAK}.

Because tyrosine phosphorylation of β -catenin increased after HGF stimulation or Src transformation, we investigated the action of PAF and its receptors on cell adherens junctions in

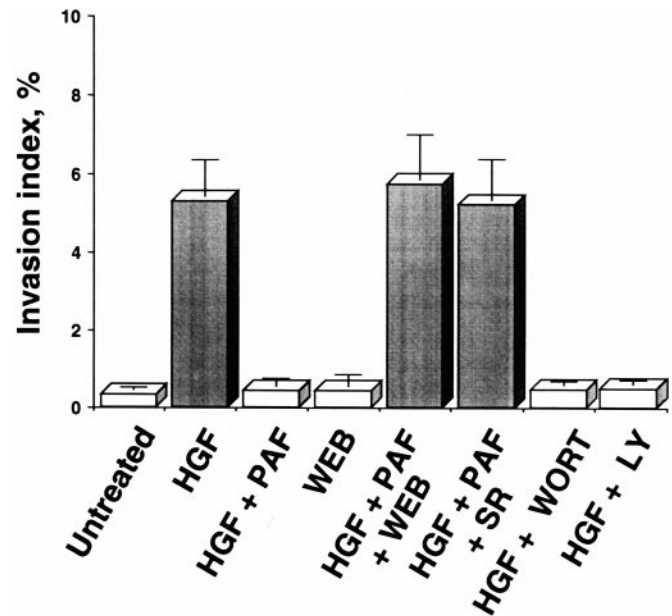


FIG. 3. Inhibitory effect of PAF and PI3'-K inhibitors on the HGF-induced invasiveness of human colonic cancer cells PCmsrc: reversion by PAF-R antagonists. PCmsrc cells were incubated for 24 h with one of the following substances or combinations: HGF (10 units/ml); HGF plus 0.1 μ M PAF, alone or combined with its PAF-R antagonists WEB2086 (WEB, 10 nM) or SR27417A (SR, 10 nM); WEB alone; or HGF plus the PI3'-K inhibitors wortmannin (WORT, 10 nM) or LY294002 (LY, 10 μ M). The percentage of invasive cells was determined as indicated under "Experimental Procedures." Data are means $\pm$ S.E. of four to eight separate experiments.

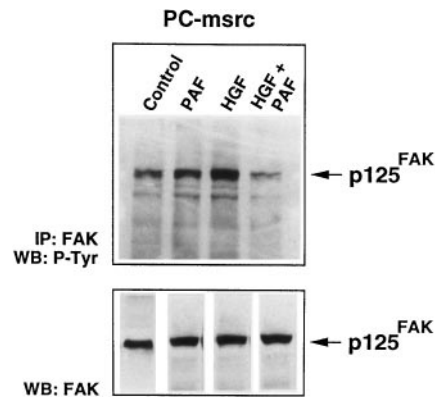


FIG. 4. Inhibitory effect of PAF on HGF-induced tyrosine phosphorylation of p125^{FAK} in the human colonic cancer cell line PCmsrc. After treatment of cells for 24 h with HGF (10 units/ml), PAF (0.1 μ M), or HGF plus PAF, whole cell lysates prepared from PCmsrc cells were immunoprecipitated with the mAb clone 77 against p125^{FAK} (Transduction Laboratories), and analyzed using the anti-phosphotyrosine mAb 4G10 (UBI). After stripping the membranes, equal expression was observed by reprobing with the same anti-p125^{FAK} mAb, clone 77. Results are representative of two other independent experiments.

our system (32). We found that PAF had no effect on tyrosine phosphorylation of the 95-kDa band corresponding to β -catenin in either control or HGF-treated ts-srcMDCK cells when they were incubated at the restrictive temperature of 40 $^{\circ}$ C (data not shown).

When kidney epithelial ts-srcMDCK cells were incubated at the restrictive temperature of 40 $^{\circ}$ C, we observed that 0.1 μ M PAF completely reversed the HGF-dependent invasiveness (Fig. 5A). The presence of specific and functional PAF-R in this model was confirmed by the observation that the PAF-R antagonists WEB2086 and SR27417A abolished the inhibitory effect of PAF on invasion. At 10 nM, the PI3-K inhibitor wort-

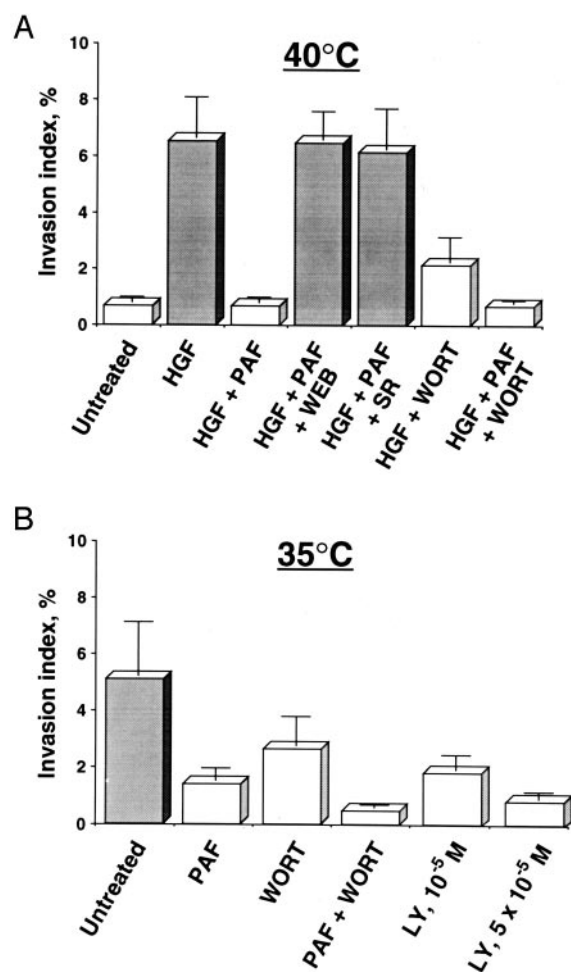


FIG. 5. Inhibitory effect of PAF and PI3'-K inhibitors on HGF- and Src-induced invasiveness of ts-srcMDCK epithelial cells: reversion by PAF-R antagonists. *Panel A*, ts-srcMDCK cells were incubated for 24 h at the restrictive temperature of 40 °C with HGF (10 units/ml) alone or combined with the following effectors: PAF (0.1 μ M); PAF and its PAF-R antagonists WEB2086 (WEB, 10 nM) or SR27417A (SR, 10 nM); the PI3'-K inhibitor wortmannin (WORT, 10 nM); or PAF plus wortmannin. The percentage of invasive cells was determined as indicated under "Experimental Procedures." Data are means $\pm$ S.E. of four or five separate experiments. *Panel B*, ts-srcMDCK cells were incubated for 24 h at the permissive temperature of 35 °C with either 0.1 μ M PAF, the PI3'-K inhibitor wortmannin (WORT, 10 nM), and the combinations PAF plus wortmannin or the PI3'-K inhibitor LY294002 at the indicated concentration. Data are means $\pm$ S.E. of four to five separate experiments.

mannin inhibited the HGF-dependent invasiveness of ts-srcMDCK cells. Expression of PAF-R in MDCK cells was further confirmed by Northern blot, after identification of the corresponding transcript of 4 kilobase pairs (data not shown).

At the permissive temperature of 35 °C, PAF and wortmannin had additive inhibitory effects on the Src-induced invasiveness of ts-srcMDCK cells (Fig. 5B). Treatment with 10 μ M LY294002 reduced this invasiveness by 63%.

Invasiveness of Parental and ts-srcMDCK Cells Transfected with Activated or Dominant-negative Forms of PI3'-K p110 α : *Measurement of PI3'-K Activity*—As shown in Fig. 6A, introduction of the constitutively activated p110 α subunit of PI3'-K to parental MDCK cells (19) induced remarkably strong invasiveness. This activated form of the enzyme potentiated the HGF-induced invasion. In contrast, activation of PAF-R abolished the invasiveness induced by activated p110* and reversed the additional invasion produced by HGF (Fig. 6A).

In agreement with these findings, we observed that 1) PAF

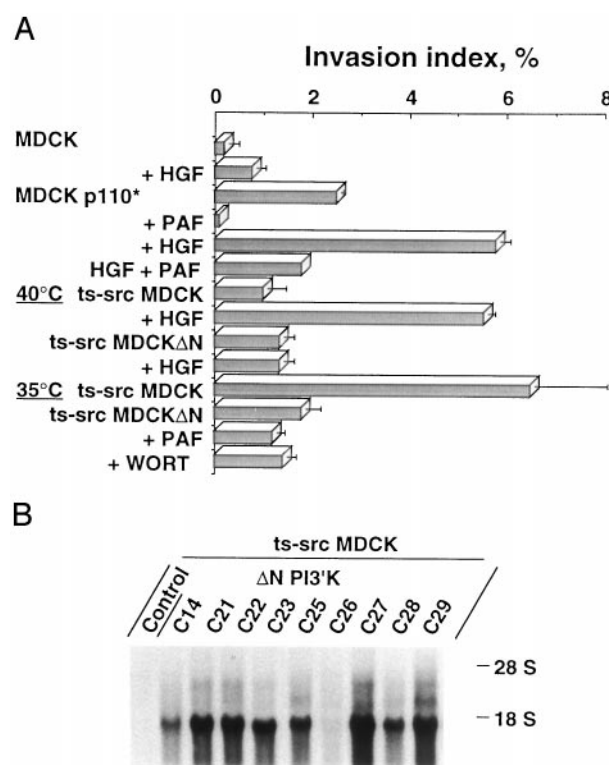


FIG. 6. Effects of constitutively activated or dominant-negative forms of PI3'-K-p110 α on spontaneous, Src-induced, and HGF-induced invasiveness of MDCK epithelial cells. *Panel A* shows (i) the induction and potentiation of spontaneous and HGF-induced invasiveness in parental and stably transfected MDCK cells by the constitutively activated p110* form of PI3'-K (MDCKp110*), (ii) the suppression of HGF- and Src-induced invasiveness by the dominant negative Eco-S form of PI3'-Kp110 α in ts-srcMDCK Δ N cells (clone 27) incubated at either 40 or 35 °C; (iii) at the permissive temperature of 35 °C, reversal by PAF and wortmannin of the invasiveness of ts-srcMDCK cells by 60–73%, but not reduction of residual activity of ts-srcMDCK Δ N cells. Data are means $\pm$ S.E. of four separate experiments. *Panel B* shows the expression by Northern blot of the truncated 1.5-kilobase pair transcript encoding the dominant-negative form Δ NP13'-K of p110 α in stably transfected ts-srcMDCK Δ N clones (C14 to C27). Control ts-srcMDCK cells and clone C26 were negative.

inhibited the HGF-induced PI3'-K activation measured in anti-phosphotyrosine immunoprecipitates prepared from ts-srcMDCK cells (Fig. 7), and 2) introduction of the dominant negative mutant p110-EcoS of PI3'-K totally blocked the HGF-dependent invasiveness of ts-srcMDCK Δ N cells (clone 27) when they were incubated at the restrictive temperature of 40 °C (Fig. 6, A and B).

Clone 27 was selected among G418-resistant ts-srcMDCK Δ N colonies by Northern blot (Fig. 6B), on the basis of the expression of the truncated 1.5-kilobase pair transcript of p110 α PI3'-K, using the 5'-EcoRI fragment of the cDNA corresponding to the N-terminal part of the kinase as a specific probe. No hybridization was observed using the same membranes hybridized with the 3'-EcoRI fragment of p110 α PI3'-K (data not shown). As controls, we observed that (i) the other Δ NP13'-K-positive clone 29 (Fig. 6B) was also resistant to HGF for invasiveness, and (ii) when the Δ NP13'-K-negative clone 26 was incubated at 40 °C with HGF, it exhibited a percentage of invasiveness similar to that of ts-srcMDCK (7.7% and 6.2%, respectively). At the permissive temperature of 35 °C, the dominant negative mutant of PI3'-K p110 α strongly inhibited Src-induced invasiveness, to a degree comparable to the inhibition observed with PAF or wortmannin (see also Fig. 5B).

Effect of Pertussis Toxin (PTx) and Rapamycin on Src- and HGF/PAF-dependent Invasiveness of Transformed Epithelial

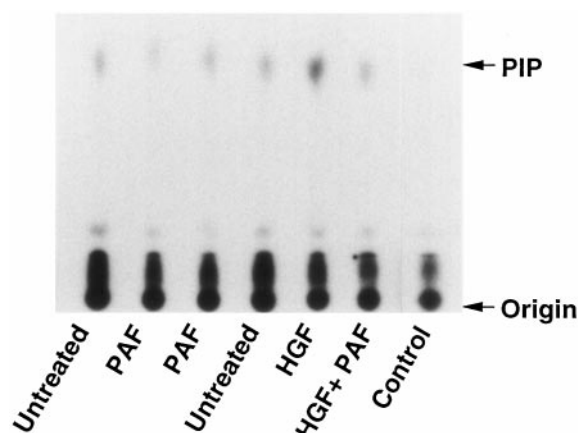


FIG. 7. *In vitro* kinase activity of PI3'-K immunoprecipitated from ts-srcMDCK epithelial cells incubated in the presence of HGF and PAF. ts-srcMDCK cells were incubated for 18 h at 37 °C in the presence of HGF (10 units/ml), PAF (0.1 μ M), HGF plus PAF, and in the absence of effector (Untreated). Then, soluble lysates were obtained for immunoprecipitation, as described under "Experimental Procedures." After extensive washing of anti-phosphotyrosine immunoprecipitates, PI3'-K activity was determined. The autoradiograph shows the radiolabeled lipid product of PI3'-K separated by thin layer chromatography with position of PI-3-P indicated. As negative control, PI3'-K determination was performed in the presence of 1% Nonidet P-40 (Control).

Cells—We next determined (Fig. 8) whether cellular responses to PAF and HGF in PCmsrc and ts-srcMDCK cells were inhibited by PTx, because the effects of PAF are mediated via the activation of serpentine G-protein-linked transmembrane receptors. We first observed that treatment of ts-srcMDCK cells for 24 h, at the permissive temperature of 35 °C with 200 ng/ml PTx, had no effect on Src-induced invasion of collagen gels (control invasion: $8.33 \pm 0.43\%$; PTx treatment: $8 \pm 0.17\%$, $n = 3$).

In contrast, PTx reversed by 50% the inhibitory effect of PAF in ts-srcMDCK cells when they were incubated at the permissive temperature of 35 °C. Under the same conditions, PTx, which is responsible for the ADP-ribosylation and inactivation of $G_{\alpha_{10}}$ subunits, caused a 62–70% decrease in HGF-induced invasiveness in PCmsrc and ts-srcMDCK cells when they were incubated at the restrictive temperature of 40 °C ($n = 6$ independent experiments, $p < 0.001$ – 0.005). The HGF-dependent invasion of ts-srcMDCK cells is sensitive to 20 ng/ml PTx; half-maximal inhibition occurred at 50 ng/ml PTx.

On the other hand, Src- and HGF/PAF-dependent invasiveness of PCmsrc and ts-srcMDCK cells was not inhibited by treatment with 20 nM rapamycin for 24 h (data not shown).

DISCUSSION

The studies described here demonstrate that normal human colonic epithelial crypts, colonic adenoma, and adenocarcinoma cell lines express functional and specific PAF receptors. A noteworthy finding in this work is the identification of a new alternative splice variant in the 5'-UTR of the PAF-R mRNA, in the form of an elongated version of the tissue-type transcript. This variant includes an additional sequence of 82 nucleotides from the 3' end of the leukocyte-type exon 1 transcript (33). It has been postulated that long 5'-UTR transcripts are involved in the formation of secondary structures that must be eliminated for the efficient translation initiation (34). Elongated forms of 5'-UTR have a potential role in the extensive interactions with multiple factors that control the rates of initiation and translation of human PAF-R mRNA in human colonic epithelial cells. Similarly, the tissue-type transcript and this new variant harbor a second ATG in exon 2. This second

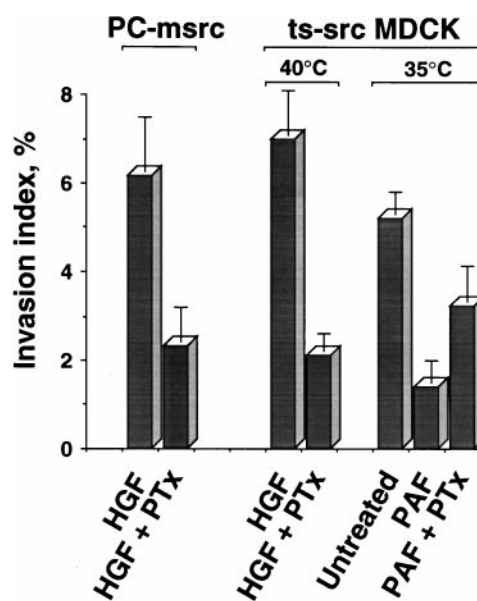


FIG. 8. Effects of PTx on HGF- and PAF-dependent invasiveness of PCmsrc and ts-srcMDCK epithelial cells. The percentage of invasive cells was measured as specified under "Experimental Procedures." Cells were treated for 24 h with the indicated effectors, in the presence or absence of PTx (200 ng/ml). Data are means $\pm$ S.E. of six separate experiments.

open reading frame, comprising 44 codons, is unusually long compared with other G-protein coupled transmembrane receptors (35), as well as to the leukocyte-type PAF-R transcript (36). The human β_2 adrenergic receptor has been shown to have a second upstream ATG and an elongated 19-codon open reading frame in the 5'-UTR. When this upstream open reading frame was deleted from the corresponding cRNA, production of the receptor protein rose about 10-fold, when translated in the reticulocyte system (37), suggesting competitive interactions between the ATG signals. A very similar feature characterizes both the cholinergic and estrogen receptors. Precise function of the elongated form of the 5'-UTR in intestinal PAF-R mRNA remains to be elucidated. Because of the large intron separating exons 1 and 2, previous studies failed to establish the organization of the PAF-R gene (23). The identification of a new tissue-type splice variant in intestinal cells enabled us to establish the physical map of the human PAF-R gene, as exon 2 was upstream of exon 1 (Fig. 1). Our data suggest that PAF can directly amplify the harmful action of inflammatory agents in human colonic epithelial cells, because this potent lipid mediator, which is generated during cell injury, triggered the expression of the inducible prostaglandin synthase gene that leads to the synthesis of inflammatory agents prostaglandins, prostacyclins, and thromboxanes. Another active mediator of the inflammatory response, such as bacterial lipopolysaccharide, induces PAF release, PAF-R expression, and tumor necrosis factor- α (38), suggesting that multiple paracrine and autocrine pathways are involved in cell injury in the intestinal mucosa.

PAF-R activation is connected with changes in cell shape, cytoskeletal reorganization, and expression of urokinase-type plasminogen activator (48). This extracellular serine protease is strongly expressed in transformed cell lines and is directly or indirectly involved in degrading matrix components, including fibrin, fibronectin, laminin, or basement membrane proteins like type IV collagen. Recent studies indicate that the complexes formed by FAK with other cellular proteins are important in the determination of its kinase activity and signaling functions in focal contacts. In immediate signal-dependent

changes, FAK is directly connected to extracellular and intracellular signals activated by growth factors, integrin receptors, matrix components, and oncogenic pathways, thus providing docking sites for a complex cluster of SH2-containing proteins and promoting functional links between the cytoskeletal protein network, cell adhesion, and migration (49). Because both PI3'-K and FAK are downstream components of activated Src and HGF/Met, these functional links led us to investigate the role of PAF and its receptors on these two signaling pathways. We found that PAF impaired the HGF-stimulated tyrosine phosphorylation of FAK that was shown to correlate with its increased kinase activity. Interestingly, we also found that the invasiveness of transformed human colonic and canine kidney epithelial cells is inhibited in a cooperative fashion by PAF-R and PI3'-K inhibitors, and is mimicked by a dominant-negative mutant of PI3'-K p110 α , thus providing evidence that activation of PI3'-K is an absolute requirement for Src- and HGF-dependent invasion of tumor cells. In agreement, PAF completely abrogated the invasiveness induced by constitutive activation of the PI3'-K p110 α catalytic subunit and inhibited the HGF-induced increase in PI3'-K activity in ts-srcMDCK cells. It was therefore evident that PAF exerts a negative regulation on this cellular activity and signaling pathway, including the downstream mediators of PI3'-K, such as the pleckstrin homology domain-dependent effectors and Rho-like small G-proteins. Alternatively, inhibition of invasion by activated PAF-R may occur through the association of PI3'-K with the p85-kDa regulatory subunit(s) or other non-tyrosine-phosphorylated proteins that influence the stability and activity of the lipid kinase at the plasma membrane. Taken together, our data indicate that the inhibitory action of PAF on invasiveness is coordinated with changes in the activities of both FAK and PI3'-K in Src-transformed epithelial cells.

Because both wortmannin and LY294002 inhibit the PI3'-K/Akt serine/threonine kinase cascade that activates the p70 ribosomal protein S6-kinase (p70^{S6k}), we next examined the effect of rapamycin on our system. This immunosuppressant indirectly blocks the phosphorylation/activation of p70^{S6k}, forming a complex with FK-506-binding protein and TOR, a lipid kinase upstream of p70^{S6k}. This pathway controls the translation of the proteins required for growth factor signaling, progression through the cell cycle, and delivers signals for cell survival and the polarized distribution of the actin cytoskeleton (50). We found that rapamycin had no effect on Src- or HGF/PAF-dependent invasiveness in PCmsrc or ts-srcMDCK cells, suggesting that the signaling elements downstream of TOR-p70^{S6k} are not involved in this invasiveness. Moreover, our present observation that HGF-induced tyrosine phosphorylation of β -catenin was not inhibited by PAF in ts-srcMDCK cells rules out the possibility that the E-cadherin/catenin complex has a functional role in PAF action. In agreement, we observed that invasiveness of ts-srcMDCK cells induced by the monoclonal antibody Degma-1 directed against murine E-cadherin is resistant to PAF (data not shown). We therefore conclude that the PAF-dependent invasiveness of transformed epithelial cells involved E-cadherin- and p70^{S6k}-independent pathways.

We presented evidence that PAF inhibits invasion via PTx-sensitive and -resistant G-proteins. Although the identity of the individual G-protein isotypes activated by human PAF-R is still a matter of conjecture, the PTx-insensitive isoforms G_s/G α_{11} /G α_q and the PTx-sensitive isoforms G α_o /G α_{i1} /G α_{i2} /G α_{i3} are probably involved (28). PTx-insensitive heterotrimeric GTP-binding proteins include (i) the ubiquitously expressed G α_q , G α_{11} , G α_{12} , and G α_{13} isotypes, which activate specific phospholipase C isoforms and cell growth signaling, and (ii) the G α_z isotype involved in adenylate cyclase inhibition. Consistent

with our finding, it has been proposed that PAF receptors trigger either PTx-resistant or -sensitive activation of signaling pathways, depending on the G-protein isotype and the effector system concerned (44).

Another interesting finding derived from the experiments described herein is that the HGF-dependent invasiveness of PCmsrc and ts-srcMDCK cells is negatively regulated by PTx. Therefore, PTx exerts opposite effects on the PAF- and HGF-dependent invasion of Src-transformed epithelial cells. This is the first evidence that Met receptors activate (or synergize with) a latent signaling pathway that utilizes a member of the G-family of heterotrimeric proteins. The association of single transmembrane protein-tyrosine kinase receptors with G-proteins was recently documented in the case of the direct interaction between the epidermal growth factor receptor juxtamembrane region and the α -subunit of G_s (51). Our future investigations will be designed to identify the PTx-sensitive G-proteins involved in the molecular and functional integration of the positive and negative signals participating in the cross-talk between PAF-R and the tyrosine kinases Src and Met in tumor invasion. In this connection, recent evidence indicates that four classes of PI3'-K catalytic subunit generate the signaling molecule phosphatidylinositol-3,4,5-trisphosphate from upstream receptors: the p110 α , β , and γ lipid kinases, and the recently discovered AGE kinase involved in regulating longevity in *Caenorhabditis elegans*. Most interestingly, the wortmannin-sensitive p110 γ isoform was strongly activated by both the α and $\beta\gamma$ subunits of heterotrimeric G-proteins and induced mitogen-activated protein kinase activation (52). One function of $\beta\gamma$ is to locate p110 γ to the plasma membrane, via a tightly associated adaptor, p101 (53). This p110 γ isoform of PI3'-K is strongly activated by the PTx-dependent G α_{i1} subunit (52), suggesting that the molecular cluster containing G α_{i1} , p110 γ , and $\beta\gamma$ -p101 is a candidate effector of the Met pathway in invasion. Consistent with these interactions, it was recently reported that heterodimeric PI3'-K consisting of p110 β and p85 is synergistically activated by the $\beta\gamma$ subunits of trimeric G proteins (54). Release of G $\beta\gamma$ also promotes tyrosine phosphorylation of Shc and its subsequent association with Grb2-SOS. Complexity of the molecular systems involving PI3'-K is further documented by the molecular and functional diversity of the p85 regulatory subunits of the PI3'-K enzyme family (55).

In conclusion, we showed that, in human colonic mucosa, PAF and its membrane receptors may be directly involved, at the epithelial cell level, in inflammatory processes and normal or neoplastic growth. Because activation and amplification of the Src and Met oncogenes are frequent events in human colon cancer and many human tumors (11), future studies should center on the downstream elements connected with PAF-R and Src/Met-R kinases. These elements include the PI3'-K cascade and the identification of its downstream targets and ultimate effectors, such as focal adhesions, cytoskeleton components, or proteases. These investigations should prove informative, in view of the possible beneficial effects of PAF derivatives against tumor cell invasion and cancer progression toward metastasis.

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Addendum—Recently Keely *et al.* (56) and Shaw *et al.* (27) made similar observations that PI3'-K is involved in carcinoma invasion induced by Rac or $\alpha_6\beta_4$ integrins.

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