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 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 p110a 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 acetylatedlases, 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 p125FAK 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 pairs; 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<sub>FAK</sub> by HGF that was completely blocked by PAF; (v) constitutively activated PI3'-K and dominant negative forms of the PI3'-K p110α 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 (Gα<sub>13/βγ</sub> 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<sup>32</sup>P]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 antiphosphotyrosine monoclonal antibody (mAb) 4G10, and the antip125<sub>FAK</sub>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 nonmotile and exhibits a mucinous phenotype (16). After transfer of the activated c-src oncogene, PC/AA/C1 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 intestinal epithelial cell lines Caco-2, PC/AA C1, CFI-3, HT-29, and Transduction Laboratories, respectively. Cells were transfected by the activated form of bovine p110*<sub>106</sub> cells (Life Technologies, Inc.). After 48 h, cells were trypsinized and plated onto 24-well plates (15). The PC/AA/C1 cell line was established from a colonic adenoma in a patient with familial adenomatous polyposis. 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 does not produce any tumor in athymic nude mice and is relatively undifferentiated (15). Northern 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 μg of total RNA, using 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), and random hexanucleotides (5 μM). A fraction of the corresponding cDNA (1/10) was then amplified in 20 μl of reaction buffer containing 200 μM amounts of each dNTP, 0.5 μM 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. Northern blot analysis was performed to verify the authenticity of the PCR products, using two internal probes: probe 1 (5'-CATCAAGACTGTCAGGCGAACA-3') and probe 2 (5'-CTCGAAGCTGGCTGCTGCT-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 gel using the Qiagen kit (Cogen, France) and cloned in a PCR-TRAP vector (GenHunter Corp.). Sequencing was performed using the Leseq 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<sub>1</sub> nylon membranes (Amersham Pharmacia Biotech, United Kingdom), and hybridized with the 32P-labeled probes (MegaPrime, Amersham Pharmacia Biotech). The relative amount of mRNA 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 p110α 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 trypt-

ic 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 2096 and SR27417A, or with one of the phospholipase C activators (1-phenyl-2-

butanol and 12-O-tetradecanoylphorbol-13-acetate). The extent 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—Nonconfluent PCmsrc 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 PCmsrc 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 (1–5 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, 10% glycerol, 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 µCi [γ⁻³²P]-ATP, and 0.4 µg/ml of a sonicated mixture of phosphatidyl-
sine and phosphatidylinositol (1:1, w/w). The tubes were incubated at 30 °C for 10 min. The reaction was terminated by addition of 100 µl of 1× 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. Leu-

kocyte-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 (αT). After cloning and sequencing, we demonstrated that the tissue-type 252-bp sequence is identical to the PAF-R transcript as reported previously (22). On the other hand, we observed that the additional tissue-type 334-bp product (αT) sequenced in Caco-2 cells was lengthened by the following 82 nucleotides at the 3′-end of exon 1, as shown in Fig. 1: ACAGCATAGGAGCTAGGCTGCTGGCCAACGGACCAGAACAGA-

AGAGACACAGGTCACTGACAGCAGGCTGCGCTGCCCGTACAG (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 αT se-

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 epithe-


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ceptor 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 (crypt), SV40LT-immortalized CFI-3 intestinal epithelial cells, the human colonic epithelial cancer cell line Caco-2 (enterocyte-like), HT29 (mainly undifferentiated), and HT29-MTX (mucin-secreting), peripheral human monocytes (mono) and their derived macrophages (macroph), 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.

Effects of HGF, PAF, and PI3'-K on Invasion and Tyrosine Phosphorylation of p125FAK 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 (p125FAK) 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 p125FAK.

As shown in Fig. 3, 0.1 μM 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 behavior of the HGF-stimulated invasion was observed by reprobing with the same anti-p125FAK mAb, clone 77. Results are representative of four to eight separate experiments.

As an extension of these studies, we observed that HGF induced a marked increase in the level of tyrosine phosphorylation of p125FAK in PCmsrc cells (Fig. 4). The anti-p125FAK 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 p125FAK.

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

When kidney epithelial ts-srcMDCK cells were incubated at the restrictive temperature of 40 °C, we observed that 0.1 μM 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-
mamn 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 μM LY294002 reduced this invasiveness by 63%.

Invasiveness of Parental and ts-srcMDCK Cells Transfected with Activated or Dominant-negative Forms of PI3'-K p110α: Measurement of PI3'-K Activity—As shown in Fig. 6A, introduction of the constitutively activated p110α 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 p110α and reversed the additional invasion produced by HGF (Fig. 6A).

In agreement with these findings, we observed that 1) PAF and its PAF-R antagonists WEB2086 (WEB, 10 μM) 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 ± S.E. of four or five separate experiments. 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 μM); PAF and its PAF-R antagonists WEB2086 (WEB, 10 μM) 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 ± 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 μM 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 ± S.E. of four to five separate experiments.

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 μM); PAF and its PAF-R antagonists WEB2086 (WEB, 10 μM) 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 ± 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 μM 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 ± S.E. of four to five separate experiments.

The expression by Northern blot of the truncated 1.5-kilobase pair transcript encoding the dominant-negative form ΔNPI3'-K of p110α in stably transfected ts-srcMDCKΔN 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-srcMDCKΔN cells. Data are means ± 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 ΔNPI3'-K of p110α in stably transfected ts-srcMDCKΔN clones (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-srcMDCKΔN colonies by Northern blot (Fig. 6B), on the basis of the expression of the truncated 1.5-kilobase pair transcript encoding the dominant-negative form ΔNPI3'-K of p110α. ts-srcMDCK, using the 5'-EcoRI fragment of the cDNA corresponding to the N-terminal region of the kinase as a specific probe. No hybridization was observed using the same membranes hybridized with the 3'-EcoRI fragment of p110α PI3'-K (data not shown). As controls, we observed that (i) the other ΔNPI3'-K-positive clone 29 (Fig. 6B) was also resistant to HGF for invasiveness, and (ii) when the ΔNPI3'-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 p110α strongly inhibited Src-induced invasiveness, to a degree comparable to the inhibition observed with PAF or wortmannin (see also Fig. 5B).
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 ± 0.43%; PTx treatment: 8 ± 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 Go 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 variant harboring a second ATG in exon 2. This second 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 beta2 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-alpha (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 fibronectin, laminin, or basement membrane proteins like type IV collagen. Recent studies indicate that the complexes formed by PAK with other cellular proteins are important in the determination of its kinase activity and signaling functions in focal contacts. In immediate signal-dependent

**FIG. 7.** In vitro kinase activity of P13′-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 μt), 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, P13′-K activity was determined. The autoradiograph shows the radiolabeled lipid product of P13′-K separated by thin layer chromatography with position of PI-3-P indicated. As negative control, P13′-K determination was performed in the presence of 1% Nonidet P-40 (Control).

**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 ± S.E. of six separate experiments.
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 p110α, 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 p110α 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 PCMsce 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 isomers Gj/Gi/Gαi and the PTX-sensitive isomers Gαj/Gαi/Gαq/Gα12 are probably involved (28). PTX-insensitive heterotrimeric GTP-binding proteins include (i) the ubiquitously expressed Gαq, Gα12, Gα13, and Gα13 isotypes, which activate specific phospholipase C isoforms and cell growth signaling, and (ii) the Gαi isotype involved in adenylylate 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 PCMsce 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 Gi (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 phosphatidyl-inositol-3,4,5-trisphosphate from upstream receptors: the p110α, β, γ and lipid kinases, and the recently discovered AGE kinase involved in regulating longevity in Caenorhabditis elegans. Most interestingly, the wortmannin-sensitive p110γ isofrom was strongly activated by both the α and βγ subunits of heterotrimeric G-proteins and induced mitogen-activated protein kinase activation (52). One function of βγ is to locate p110γ to the plasma membrane, via a tightly associated adaptor, p101 (53). This p110γ isofrom of PI3'-K is strongly activated by the PTX-dependent Goq subunit (52), suggesting that the molecular cluster containing Goqγ, p110γ, and βγ-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 p110β and p85γ is synergistically activated by the βγ subunits of trimeric G proteins (54). Release of GoGβγ 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 colon 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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