Human tissue factor pathway inhibitor-2 (hTFPI-2) is a 32-kDa serine protease inhibitor that is associated with the extracellular matrix. hTFPI-2 inhibits several extracellular matrix-degrading serine proteases and may play a role in tumor invasion and metastasis. To study the signal transduction pathway that leads to the activation of hTFPI-2, we cloned the potential promoter region of this gene adjacent to a heterologous luciferase reporter gene. Phorbol 12-myristate 13-acetate (PMA) induced the luciferase reporter gene in HEK293 cells and other epithelial cell lines, such as the human lung carcinoma A549 cells, the breast carcinoma MCF7 cells, and the cervical HeLa cells. This PMA induction was blocked with the MEK inhibitor U0126, suggesting that the PMA-induced activation of the hTFPI-2 promoter is mediated through MEK. Furthermore, epidermal growth factor induced the luciferase reporter gene in HEK293 cells. Cotransfection of the luciferase construct with constitutively active components of the Ras/Raf/MEK/ERK pathway in EcR-293 cells lead to a 7- to 92-fold induction of the luciferase reporter gene, indicating that regulation of hTFPI-2 is mediated through this pathway. A series of luciferase reporter gene constructs with progressive deletions of the 5′-flanking region suggested that the minimal basal promoter activity is located between nucleotide positions −89 and −384, whereas the minimal inducible promoter activity is between −89 and −222. We have used the computer program TRANSFAC and mutagenesis to analyze potential transcription factor binding sites. We identified an AP-1 binding site at nucleotide position −156 (inducible activity) and a Sp1 site at position −134 (basal activity) as potential cis-acting elements in the promoter region of the hTFPI-2.

Growth hormones and the tumor promoting agent PMA initiate diverse intracellular signaling pathways that lead to the phosphorylation of transcription factors and ultimately to the regulation of target genes. Among the pathways often used to transduce signals are the mitogen-activated protein kinase (MAPK) cascades. These cascades consist of a three-kinase module that includes an MEK kinase (MEKK), which activates an MAPK/ERK kinase (MEK), which in turn activates a MAPK (1). Three well characterized MAPKs have been described in mammalian cells: the mitogen-responsive ERK, the stress-responsive JNK/SAPK, and the p38 MAPK. The Ras/Raf/MEK/ERK signaling cascade regulates cell proliferation and differentiation (2). Components of this pathway are often activated in human tumors and oncogenic Ras, and constitutively activated ERKs have been found in a large variety of malignancies (3–5). We have used transcript profiling to identify genes that are differentially regulated by this pathway. Among the many activated genes, we have identified the human tissue factor pathway inhibitor-2 (hTFPI-2) as a gene that is highly up-regulated by the ERK/MAPK pathway.

hTFPI-2 is a 32-kDa serine proteinase inhibitor with three tandem Kunitz-type domains (6, 7) and has high homology to hTFPI-1, a regulator of the extrinsic blood coagulation pathway. The second Kunitz-type domain of hTFPI-1 binds to factor Xa, and this complex inhibits the activity of the factor VIIa-tissue factor complex through interaction of the first Kunitz-type domain in hTFPI-1 and the active site of VIIa/TF (8). Despite the high homology of hTFPI-2 to hTFPI-1, hTFPI-2 is a weak inhibitor of the activation of factor X (9) and hTFPI-2 poorly inhibits tissue factor. However, hTFPI-2 inhibits the tissue factor-factor VIIa complex and a variety of serine proteases, including trypsin, plasmin, plasma kallikrein, chymotrypsin, and cathepsin G, but it does not inhibit thrombin, urokinase-type plasminogen activator, and tissue-type plasminogen activator (6, 9). Most of the hTFPI-2 expressed in dermal fibroblasts and endothelial cells localizes within the extracellular matrix, probably bound to heparan sulfate (10–12). hTFPI-2 can prevent the conversion of Pro-MMP-1 (matrix metalloprotease 1, interstitial collagenase) and Pro-MMP-3 (matrix metalloprotease 3, stromelysin-1/transin-1) into MMP-1 and MMP-3 by plasmin and trypsin (13) and therefore might indirectly regulate matrix proteolysis and connective tissue turnover.

The role of hTFPI-2 in cancer progression is not completely elucidated. On one hand, hTFPI-2 has an anti-invasive effect that might be mediated via inhibition of plasmin that activates proteases promoting degradation of the extracellular matrix and tumor invasion. Several tumor cell lines were less invasive when they were stably transfected with hTFPI-2 cDNA (14–17). On the other hand, hTFPI-2 has been shown to have a pro-invasive effect in hepatocellular carcinoma cells (18). In this study, we investigated the signaling pathway and transcriptional elements that regulate the expression of the hTFPI-2 gene in epithelial cells. Although it has been shown that PMA can stimulate hTFPI-2 gene expression in glioma metastatic cells, the signaling pathways in epithelial cells are unknown.

The ERK/MAPK Pathway Regulates the Activity of the Human Tissue Factor Pathway Inhibitor-2 Promoter*
cells and that the promoter region—312 to +1 is critical for minimal and inducible promoter activity of hTFPI-2 (19), the signal transduction pathway by which PMA induces gene expression of hTFPI-2 and the promoter elements involved in hTFPI-2 regulation have not been studied in detail. Here we show that the hTFPI-2 gene expression is regulated by the ERK/MAPK signaling pathway and that the activity of this pathway is directed to an AP-1 site in the promoter of the hTFPI-2 gene.

EXPERIMENTAL PROCEDURES

Materials—The Phorbol 12-myristate 13-acetate was ordered from Sigma-Aldrich Chemicals Co. (St. Louis, MO) and recombinant human epidermal growth factor from Austral Biologicals (San Ramon, CA). The LipofectAMINE Plus reagent as well as the Ecdysone-Inducible Mammalian Expression system, including EcR-293 cells, zeocin, and pronase E from American Type Culture Collection (ATCC, Rockville, MD). The plasmid was digested with the restriction enzyme EcoRI and cloned into the pXP2 fragment. The DNA was ethanol-precipitated and dissolved in TE, pH 8.0. A 5'-kb fragment of the 5'-flanking region of the hTFPI-2 was amplified with an Expand High Fidelity PCR system (Roche Molecular Biochemicals) using the oligonucleotides 0-7 and 0-8 (Table I) to create a KpnI site. The PCR products were verified by sequencing and subcloned as KpnI/BglII sites of pIND.

Aplikation of the hTFPI-2 Promoter Region—Genomic DNA was isolated from 293 cells. The cells were washed in PBS, lysed in a buffer containing 10 mM NaCl, 10 mM EDTA, 0.5% Sarkosyl, and 10 mM Tris, pH 8.0, and incubated with proteinase K (10 mg/ml) at 50 °C overnight. After two phenol and two chloroform extractions, the genomic DNA was ethanol-precipitated and dissolved in TE, pH 8.0. A 5'-kb fragment of the 5'-flanking region of the hTFPI-2 was amplified with an Expand High Fidelity PCR system (Roche Molecular Biochemicals) using the oligonucleotides 0-7 and 0-8 (Table I) to create a KpnI and a BglII restriction site (printed in boldface). This KpnI/BglII fragment was cloned into the plasmid pX2, which contains the firefly luciferase reporter (21) and was a generous gift from Mark Featherstone, McGill University, Montreal, Canada. The resulting construct was named p1511-luc. The sequence was verified by sequencing and cloned into the SmaI/BglII site of pX2.

Mutagenesis of Single Potential Transcription Binding Sites—The putative Sp1 transcription factor binding site GGGGCGG between nucleotide positions −180 and −184 was changed to GGGGGGA, the putative AP-1 site TGAATCA between nucleotide positions −162 and −156 was altered to GGTAGCA, and the overlapping Sp1/AP-2 and GC box GCCCTGGCCGCGGCGG between nucleotide positions −144 and −126 was modified to GCCCTGCGCGCGGCGG. Double-stranded oligonucleotides containing the corresponding nucleotide changes were phosphorylated, annealed, and cloned as HindIII/EcoRI fragments into the corresponding sites of p1511-luc. Each mutation was confirmed by DNA sequencing. The oligonucleotides used for p198MSPIB1/2-luc were O-9 and O-10, for p198MAP2-luc O-11 and O-12, and for p198MSPIB1/2-luc O-13 and O-14 (Table I). The PCR products were verified by sequencing and subcloned as HindIII/EcoRI fragments into the corresponding sites of p1511-luc. Each mutation was confirmed by DNA sequencing. The oligonucleotides used for p198MSPIB1/2-luc were O-9 and O-10, for p198MAP2-luc O-11 and O-12, and for p198MSPIB1/2-luc O-13 and O-14 (Table I). The PCR products were verified by sequencing and subcloned as HindIII/EcoRI fragments.

**Table I**

| Oligonucleotides |
|------------------|
| O-1              |
| O-2              |
| O-3              |
| O-4              |
| O-5              |
| O-6              |
| O-7              |
| O-8              |
| O-9              |
| O-10             |
| O-11             |
| O-12             |
| O-13             |
| O-14             |
| O-15             |
| O-16             |
| O-17             |
Luciferase activity was calculated as light units/µg of protein and normalized for transfection efficiency. Data are shown as mean ± S.D. from one representative experiment performed in duplicate transfections. B, inhibition of Phospho-p44/42 MAPK by UO126. HEK293 cells were incubated with 250 nM PMA for 4 h (lane 1) and treated with 0.1, 1, or 10 µM UO126 prior to PMA treatment (lanes 2–4). Proteins were separated on a 12% SDS-PAGE and subjected to Western blotting with the Phospho-p44/42 MAPK (Thr202/Tyr204) antibody.

**Fig. 1.** Induction of the hTFPI-2 promoter activity by PMA is inhibited by the MEK inhibitor UO126. A, HEK293 cells were transiently cotransfected with the luciferase reporter plasmid p-1511-luc and a control vector containing GFP. 24 h later, the cells were serum-starved for 24 h. UO126 was added at a concentration of 10 or 100 µM 15 min prior to 250 nM PMA treatment for 4 h. Luciferase activity was calculated as light units/µg of protein and normalized for transfection efficiency. Data are shown as mean ± S.D. from one representative experiment performed in duplicate transfections. B, inhibition of Phospho-p44/42 MAPK by UO126. HEK293 cells were incubated with 250 nM PMA for 4 h (lane 1) and treated with 0.1, 1, or 10 µM UO126 prior to PMA treatment (lanes 2–4). Proteins were separated on a 12% SDS-PAGE and subjected to Western blotting with the Phospho-p44/42 MAPK (Thr202/Tyr204) antibody.

**Transient Transfection—**For inhibitor studies, the HEK293 cells (150,000 cells/well) were seeded the day prior to transfection. The cells were cotransfected with 1 µg of p-1511-luc and 70 ng of a GFP-spectrin control plasmid (23). 24 h after transfection, the cells were serum-starved for 24 h. Where indicated, the MEK1/2-specific inhibitor UO126 was added 15 min prior to PMA induction. The cells were harvested in phosphate-buffered saline (PBS) and divided into two tubes. Cells in one tube were lysed using cell culture lysis reagent (Promega) and centrifuged at 12,000 × g for 5 min, and the cell extract was assayed for firefly luciferase activity using the luciferase reporter assay system (Promega). Light intensity was measured by using a microtiter plate luminometer (DYNEX Technologies, Inc., Chantilly, VA). The cells in the second tube were trypsinized and washed, and the percentage of cells that express GFP was determined by cytofluorimetry (EPICS XL-MCL) to control for transfection efficiency. Determination of protein by the Bradford assay (Bio-Rad) was carried out, controlling for harvesting efficiencies. Luciferase activity was expressed as firefly light units/µg of protein and normalized for transfection efficiency.

HEK293, HeLa, A549, and MCF7 cells were seeded into six-well plates at 150,000, 250,000, 250,000, and 400,000 cells/well, respectively, 1 day prior to transfection. All cells were transfected with 1 µg of the reporter gene constructs. HEK293 and HeLa cells were transfected with FuGENE6 at a 3:1 ratio of reporter plasmid, 0.1 µg of p-1511-luc and 70 ng of a GFP control plasmid). 24 h after transfection, the cells were serum-starved overnight and subsequently treated with PMA (250 nM) or EGF (50 ng/ml) as indicated. Cell extracts were lysed and analyzed as described above. The fold stimulation of luciferase was calculated as firefly light units/µg of protein of PMA- or EGF-treated cells divided by the firefly light units/µg of protein of nontreated cells.

Two days prior to transfection, EcR-293 cells cultivated in DMEM supplemented with 10% fetal bovine serum and Zeocin (400 µg/ml) were seeded into six-well plates at 200,000 cells/well. EcR-293 cells stably express the modified edcdysone receptor. A gene of interest, cloned into a pIND-based inducible expression vector, can be induced with the edcdysone analog pronasteron A that binds to the edcdysone receptor. Cells were transfected with 6 µl of FuGENE6 and 1.07 µg of total DNA (0.9 µg of RasV12, RafCT, MEK-1SD, ERK2-MEK1-LA, or pIND control vector, 0.1 µg of reporter plasmid, and 70 ng of GFP control plasmid). The medium containing fetal bovine serum was replaced 24 h after transfection with DMEM without serum, pronasteron A was added 4 h later to the medium to induce protein expression at a concentration of 6 µM unless indicated differently, and cells were harvested 20 h later. Luciferase activity was expressed as firefly light units/µg of protein and normalized for transfection efficiency.

**Western Blots—**Cells were harvested and washed with PBS. The cells were lysed in PBS containing 0.1 mM sodium vanadate, 10 mM sodium pyrophosphate, 1.5% Triton X-100, and the proteinase inhibitors aprotonin (10 µg/ml), leupeptin (10 µg/ml), and phenylmethylsulfonyl fluoride (1 mM). 15 µg of total protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 1% bovine serum albumin in TBST (150 mM NaCl, 0.05% Tween 20, 20 mM TrisCl, pH 7.5) and subsequently incubated with the antibodies (9E10) at a dilution of 1:250 at 4 °C overnight, or with anti-Raf-1 (C12), or anti-MEK1 (C18) at dilutions of 1:1000 for 1 h at room temperature. The Phospho-p44/42 MAPK (Thr202/Tyr204) antibody was diluted 1:1000 in TBST containing 4% skin milk powder and incubated for 1 h. After incubation with the secondary antibodies conjugated to horseradish peroxidase (1:1000) for 1 h, the bands were visualized by ECL (Roche Diagnostics Corp., Indianapolis, IN).
RESULTS

We have observed that the hTFPI-2 gene was highly up-regulated on microarrays that were probed with cDNAs originating from Hek293 cells treated with 250 nm PMA for 4 h (data not shown). Recently, PMA induction of a hTFPI-2-luciferase reporter gene has also been shown in glioma cells (19), and mRNA of hTFPI-2 is up-regulated in BeWo and JEG-3 trophoblast cells treated with PMA (24).

To study the signaling pathway that leads to the induction of hTFPI-2 gene expression by PMA and to investigate important promoter elements involved in its transcriptional regulation, we PCR-amplified a 1511-bp fragment of the 5′-flanking region of the hTFPI-2 gene from genomic DNA isolated from Hek293 cells and cloned the 1511-bp fragment adjacent to the firefly luciferase reporter gene (p-1511-luc).

hTFPI-2 Promoter Activity Induction by Phorbol Esters and Inhibition of the PMA-dependent Induction by the MEK Inhibitor UO126—To assess whether this potential promoter region allows transcription of luciferase, we transiently transfected Hek293 cells with the reporter plasmid p-1511-luc and monitored changes in luciferase activity of PMA-treated and untreated cells. Cells incubated with 250 nm PMA showed a 10-fold stimulation of luciferase compared with cells without PMA treatment (Fig. 1A). Induction of the luciferase reporter gene was decreased by 90% if cells were preincubated with the MEK1 inhibitor UO126 at a concentration of 10 μM. UO126 completely inhibited PMA induction at a concentration of 100 μM, suggesting that PMA activates the hTFPI-2 promoter through the MEK signaling pathway (Fig. 1A).

As shown in the Western blot in Fig. 1B, PMA activated p44/p42 MAPK in Hek293 cells and the phosphorylation of p44/p42 could be inhibited by the MEK1-inhibitor UO126 in a dose-dependent manner. This result indicates that MEK activation is necessary for the induction hTFPI-2 promoter activity by PMA.

Phorbol Esters and the Growth Factor EGF Can Stimulate the Luciferase Reporter Gene in Epithelial Carcinoma Cell Lines—Hek293 cells have very recently been reported as atypical epithelial cells and may originate from neuronal cells (25). To determine whether the PMA induction of the hTFPI-2 gene observed in Hek293 cells was unique to this cell line or whether PMA promotes up-regulation of the hTFPI-2 gene in other epithelial cell lines as well, we transiently transfected human lung carcinoma A549 cells, breast carcinoma MCF7 cells and cervical carcinoma HeLa cells with the luciferase reporter plasmid p-1511-luc. A 3.7-fold PMA-dependent induction of the hTFPI-2 promoter activity was observed in A549 cells, whereas PMA induced the hTFPI-2 promoter activity in MCF7 and HeLa cells 30- or 20-fold, respectively (Fig. 2). Because PMA has been reported to transactivate the epidermal growth factor receptor (26), we tested whether the hTFPI-2 gene could be induced by the growth factor EGF. Although EGF up-regulated the hTFPI-2 promoter activity in HeLa cells 10-fold, no substantial stimulation of the luciferase reporter gene was obtained in A549 and MCF7 cells (Fig. 2).

Ras, Raf, MEK, and ERK Can Induce the hTFPI-2 Promoter Activity—To assess the importance of the components of the ERK/MAPK signaling pathway in the regulation the hTFPI-2 promoter activity, we cotransfected EcR-293 cells with the plasmid p-1511-luc and vectors containing constitutively activating signaling components, such as RasV12, RafCT, MEK-1SD, ERK2-MEK1-LA, or the empty control vector (pLND). The -fold stimulation of luciferase was calculated as normalized luciferase activity obtained in cells expressing active signaling components divided by the luciferase activity of samples originating from vector-transfected control cells (Fig. 3A). Protein expression of RasV12, RafCT, MEK-1SD, and ERK2-MEK1-LA in transiently transfected EcR-293 cells is shown on Western blots in Fig. 3B. All the constitutively activated signaling components were well expressed. Expression of RasV12 stimulated the luciferase reporter gene 37-fold compared with the control vector. Expression of constitutively active signaling MAPK components further downstream of Ras, such as RafCT, MEK-1SD, and ERK2-MEK1-LA, induced the luciferase reporter the gene 7-92-, or 39-fold, respectively (Fig. 3A), indicating that the hTFPI-2 gene expression can be regulated by the Ras/Raf/MEK/ERK pathway in EcR-293 cells. The highest activation of the hTFPI-2 promoter was obtained by MEK-1SD containing aspartic acids at amino acid positions 218/222 (27), whereas the RasV12 and ERK2-MEK1-LA fusion protein (28) activated the hTFPI-2 promoter to a similar extent. In conclusion, these results demonstrate that the Ras/Raf/MEK/ERK signaling pathway mediates regulation of the hTFPI-2 gene.

The Minimal Inducible Promoter Activity Is Located within the −89/−222-bp Region of the hTFPI-2 Promoter—We transiently cotransfected EcR-293 cells with a series of luciferase reporter gene constructs containing progressive deletions of the 5′-flanking region with either a vector containing RasV12 or the empty control vector pLND. The -fold stimulations of luciferase were obtained with constructs p-1511-luc through p-222-luc, whereas no inducible luciferase activity was obtained with construct p-89-luc (Fig. 4A). 35- to 51-fold stimulations of luciferase were obtained with constructs −89/−222 bp region that are responsible for the 51-fold Ras and 8-fold PMA stimulations as compared with control cells.

The minimal basal promoter activity includes the −89/−384-bp region, because p-384-luc showed similar basal luciferase activity as longer constructs, whereas no basal activity was obtained with construct p-89-luc (Table II). As shown in Table II, the luciferase activity dropped by 74% in vector (pLND)-transfected cells expressing p-222-luc compared with cells expressing p-384-luc, indicating that the region, −384 to
−222 bp, contains transcription factor binding sites important for basal activity. Similarly, luciferase light units were higher in samples originating from cells transfected with RasV12 and a reporter gene construct containing 384 bp of an upstream promoter segment compared with cells transfected with RasV12 and a reporter construct containing only a 222-bp promoter segment (Table II). This loss of activity is similar to the loss of basal activity in the −222/−384 region and therefore may be due to the absence of transcription factor binding sites important for basal activity. However, we cannot rule out that the region, −384/−222 bp, may contain additional enhancer elements that can contribute to the inducible activity.

Identification of an AP-1 Site as a Specific Inducible DNA Response Element—To determine potential cis-acting elements responsible for the PMA and Ras inducibility of the −89/−222-bp promoter region, we used the computer program TFBSEARCH version 1.3 that searches highly correlated sequence fragments versus the TFMATRIX transcription factor binding site profile data base by E. Wingender, R. Knueppel, P. Dietze, and H. Karas (GBF-Braunschweig). Several putative transcription factor binding sites were identified, including a potential Sp1 site (CCCGG) between nucleotide positions −192 and −179, a potential AP-1 site (ATGAAAT) between positions −163 and −156, and an overlapping Sp1 (underlined)/AP-2 and GC box (GGCTCCGCCCTGCGCGGCGGGGG) between positions −144 and −126 (Fig. 5).

To assess the importance of these sites, we mutated the presumptive Sp1 site (GG at position −185/−184 mutated to AA, printed in boldface above), mutated the presumptive AP-1 site (TGAAT at positions −162 to −158 mutated to GCTAG), or altered the presumptive Sp1 and AP-2 sites (CC at positions −140/−139 mutated to TT, and GG at positions −133/−132 mutated to AA; printed in boldface above). The resulting reporter gene plasmids p-198MSP1A-luc, p-198MAP1-luc, and p-198MSP1B/MAP2-luc lacked the consensus −184 Sp1A site, the consensus −156 AP-1 site, or the consensus −134/−126 Sp1B/AP-2 sites, respectively. As illustrated in Fig. 6, mutation of the putative Sp1A site in a 198-bp 5′-flanking promoter region resulted in a 40% reduction in the basal and inducible activity as compared with p-222-luc. The reporter construct p-198MSP1A was still 52-fold induced by RasV12 as compared with the control vector (Table II). The loss of some basal activity might be due to the mutation of the consensus Sp1A site or the absence of the −198 to −222 region. Mutation of the consensus AP-1 site caused a considerable decrease in inducible activity, whereas substantial basal activity was still retained, indicating that this putative AP-1 site is important for inducible activity. Furthermore, mutation of the overlapping consensus...
independent experiments performed in triplicates. The fold stimulation of luciferase was calculated as firefly light units/mg of protein of cells expressing signaling components divided by the firefly light units/mg of protein of cells transfected with the control vector pIND. Data are shown as mean ± S.D. from at least three independent transfection experiments. B, HEK293 cells were transiently transfected with the deletion constructs. The cells were serum-starved for 16 h prior to treatment with 250 nM PMA for 4 h. The fold stimulation of luciferase was calculated as firefly light units/mg of protein of PMA-treated cells divided by the firefly light units/mg of protein of mock treated cells. Data are shown as mean ± S.D. from at least three independent experiments performed in triplicates.

**TABLE II**

Luciferase activity of the deletion constructs described in Fig. 4 (normalized firefly units/mg of protein)

| Construct          | pIND  |
|--------------------|-------|
| p-1511-luc         | 11466 ± 1463 |
| p-1293-luc         | 10020 ± 2035 |
| p-1055-luc         | 10162 ± 1868 |
| p-881-luc          | 9317 ± 1734 |
| p-733-luc          | 19490 ± 3496 |
| p-384-luc          | 10702 ± 3023 |
| p-222-luc          | 4436 ± 2631 |
| p-198MSP1A-luc     | 2659 ± 619  |
| p-89-luc           | 1 ± 0.3    |

Fig. 4. Deletion constructs of the hTFPI-2 promoter. A, RasV12 or control vector pIND and luciferase reporter gene constructs with 5′-ends between nucleotides −1511 and −89 and a common 3′-end at −1 were transiently cotransfected into EcR-293 cells. A vector containing GFP was included to control for transfection efficiency. The cells were serum-starved 4 h prior to induction of protein expression with propionateron A for 20 h. The fold stimulation of luciferase was calculated as normalized firefly light units/mg of protein of cells expressing signaling components divided by the normalized firefly light units/mg of protein of cells transfected with the control vector pIND. Data are shown as mean ± S.D. from at least three independent transfection experiments. B, HEK293 cells were transiently transfected with the deletion constructs. The cells were serum-starved for 16 h prior to treatment with 250 nM PMA for 4 h. The fold stimulation of luciferase was calculated as firefly light units/mg of protein of PMA-treated cells divided by the firefly light units/mg of protein of mock treated cells. Data are shown as mean ± S.D. from at least three independent experiments performed in triplicates.

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| p-89-luc           | 1 ± 0.3    |

Fig. 5. Potential transcription factor binding sites in the minimal inducible promoter of hTFPI-2. Potential cis-acting elements as determined by TFSEARCH are underlined. Sites that were mutated are shown in boldface, and the positions are indicated by numbers.

hTFPI-2 promoter strengthened our previous observation. p-222MAP1/MSP1B-luc showed no basal activity above background and was also not stimulated by RasV12, suggesting that these sites are cis-acting elements critical for basal and inducible activity (Fig. 6). Similar results were obtained with another MAPK signaling pathway component MEK-1SD or with PMA (data not shown).

Taken together these results provide evidence that the AP-1 site at position −156 to −162 represents a cis-acting element that is critical for induction of the hTFPI-2 promoter activity by the MEK signaling pathway and the Sp1 site at position −134 to −140 is essential for basal promoter activity.

**DISCUSSION**

A schematic summary of the results presented in this study is shown in Fig. 7. We showed that the hTFPI-2 gene is up-regulated in several epithelial cells following stimulation by PMA (Fig. 2). Additionally, EGF, a growth factor, was able to stimulate promoter activity of the hTFPI-2 gene as shown in HeLa cells (Fig. 2). In HEK293 cells, induction of the promoter activity by PMA could be blocked by the MEK-specific inhibitor UO126 (Fig. 1), suggesting that PMA induction of hTFPI-2 is mediated through a pathway that involves MEK. Indeed, activated Ras, Raf, MEK, and ERK were able to promote gene transcription (Fig. 3), indicating that the Ras/Raf/MEK/ERK signaling pathway is necessary for promoter activation. The ERK/MAPK pathway activates Fos proteins, which dimerize with Jun proteins and bind as the AP-1 complex to a consensus DNA sequence 5′-TGA(G/C)TCA-3′ (29). We located an AP-1 consensus site at position −156 to −162 as a cis-acting element essential for inducible promoter activity. Additionally, a Sp1 consensus site at positions −134 to −140 of the hTFPI-2 promoter was essential for basal promoter activity (Fig. 6).

Our study in HEK293 cells indicates that the hTFPI-2 gene is regulated by the Ras/Raf/MEK/ERK signaling pathway. Each constitutively active signaling component of this pathway was able to induce high transcriptional activity of the hTFPI-2 gene. In agreement with these reporter gene studies, we observed up-regulation of the hTFPI-2 gene in preliminary microarray experiments comparing EcR-293 cells expressing
be attributed to nonkinase phorbol ester receptors such as the α- and β-chimaerins or to Ras-GRP, a phorbol ester receptor that plays a role in PMA activation of Ras (38). Furthermore, PMA has been shown to transactivate the epidermal growth factor receptor in mouse epidermal JB6 cells, and PMA-induced tumor promotion may be partially mediated through this receptor (26). PMA can activate distinct groups of MAPKs, such as the mitogen-responsive ERKs (extracellular signal-regulated kinases), the stress-responsive JNK/SAPKs (c-Jun amino-terminal kinase/stress-activated protein kinases), and p38 MAPKs. The pathways induced by PMA are cell line-dependent; for example PMA activates the JNK/MAPK pathway in normal oral keratinocytes but not in immortalized/transformed keratinocytes, HeLa cells, or HEK293 cells (39, 40). In this report we provide evidence that hTFPI-2 gene expression induced by PMA is regulated through the Ras/Raf/MEK/ERK signaling pathway. The specific MEK inhibitor UO126 could block PMA induction, indicating that MEK/ERK activation is essential for induction of hTFPI-2 promoter activity by PMA. Because UO126 inhibited PMA promoter activation at the commonly used concentration of 10 μM, we hypothesize that the ERK/MAPK pathway may be sufficient for PMA induction of hTFPI-2. Besides affecting the PMA-dependent hTFPI-2 promoter activity, UO126 had a minor negative effect on basal levels of the hTFPI-2 gene transcription, probably due to the low level of activation of hTFPI-2 by endogenous MAPK signaling components.

Activation of the ERK/MAPK causes induction of fos genes through phosphorylation of ternary complex factors (41). Indeed, we have observed up-regulation of FosB when PMA-induced HEK293 cells were probed on microarrays (data not shown). Fos heterodimerizes with Jun to form the AP-1 complex, which activates gene transcription by binding to the AP-1 element. Furthermore, Jun/Fos heterodimers can lead to increased c-Jun transcription through binding to the AP-1 sites in the c-jun promoter (41). In agreement with this, we located a putative AP-1 site at position −156 to −162 as a cis-acting element essential for inducible promoter activity.

Our results suggest that basal promoter activity is located in the region, −384 to −89 bp, of the hTFPI-2 promoter consistent with a previous promoter deletion study in human transformed bone marrow microvascular endothelial cells (22), identifying an 85-bp fragment (corresponding to the −299 to −214 region in our study) that contained most of the basal activity. In addition to this, we have identified a consensus Sp1 site at −134 to −140 that is essential for the basal activity in the promoter region, −222 to −89 bp.

The region, −222 to −89 bp, is sufficient for a 52-fold Ras and an 8-fold PMA induction of the hTFPI-2 promoter activity
as compared with basal level (Fig. 4). We therefore investigated the candidate transcription factor binding sites in this promoter region and found that the consensus AP-1 site at position −156 to −162 is essential for induction of promoter activity by Ras (Fig. 6). Recently, Konduri (19) identified a 231-bp region between −312 and −81 in the hTFPI-2 promoter region that is responsive to PMA. Investigating hTFPI-2 promoter regulation in glioma cells, Konduri found a strong repressor in the region between −927 to −1181 and enhancer elements between −1511 and −1181. These repressor and enhancer elements found in glioma cells might be tissue-specific regulatory elements, because we did not find such elements in HEK293 cells. Similar luciferase activities were observed in constructs of var-

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