The ERK/MAP kinase pathway regulates the activity of the human tissue factor pathway inhibitor-2 (hTFPI-2) promoter

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Running title: hTFPI-2 is regulated by the ERK/MAP kinase pathway

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Summary:

Human tissue factor pathway inhibitor-2 (hTFPI-2) is a 32kDa 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. In order to study the signal transduction pathway that leads to the activation of the 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 HEK 293 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 MEK1 inhibitor U0126, suggesting that the PMA-induced activation of the hTFPI-2 promoter is mediated through MEK. Furthermore, epidermal growth factor (EGF) induced the luciferase reporter gene in HeLa cells. Cotransfection of the luciferase construct with constitutively active components of the Ras/Raf/MEK/ERK pathway in EcR-293 cells lead to a 7-92 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 position –89 and –384, while the minimal inducible promoter activity is between –89 and –222. We have used the computer program TFSEARCH 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.
Introduction:

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 a MEK kinase (MEKK), which activates a MAPK/ERK kinase (MEK), which in turn activates a MAPK (1). Three well characterized MAP kinases have been described in mammalian cells: the mitogen-responsive ERK, the stress-responsive JNK/SAPK and the p38 MAP kinase. 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, Raf-1 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 upregulated by the ERK/MAP kinase 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 kalikrein, chymotrypsin, 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 ProMMP-1 (matrix metalloprotease 1, interstitial collagenase) and ProMMP-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 (14-17). On the other hand, hTFPI-2 has been shown to have a proinvasive effect in hepatocellular carcinoma cells (18).

In this study, we investigated the signaling pathway and transcriptional elements that regulate the expression of hTFPI-2 in epithelial cells. Although it has been shown that PMA can stimulate hTFPI-2 expression in glioma 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 hTFPI-2 expression is regulated by the ERK/MAPkinase signaling pathway and that the activity of this pathway is directed to an AP-1 site in the promoter of hTFPI-2.
Experimental Procedures:

Materials:

The Phorbol 12-myristate 13-acetate was ordered from Sigma-Aldrich Chemicals Co., St. Louis MO, USA and recombinant human epidermal growth factor from Austral Biologicals, San Raman CA, USA. The lipofectamine plus reagent as well as the Ecdysone-Inducible Mammalian Expression System including EcR-293 cells, zeocin and pronasteron A and the expression vector pIND were purchased from Invitrogen Corporation, Carlsbad, CA, USA. The MEK Inhibitor UO126 and the Luciferase Assay System were obtained from Promega Corporation, Madison, WI, USA. The Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody was purchased from Cell Signaling Technology, Beverly, MA USA. The c-Myc (9E10) monoclonal antibody and the MEK-1 (C-18) and Raf-1 (C-12) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The QuickChange™ Site-Directed Mutagenesis Kit was from Stratagene, La Jolla, CA, USA. The FuGENE6 transfection Reagent was obtained from Roche Diagnostics Corporation, IN, USA.

Cell culture:

The HEK293, HeLa, A549 and MCF7 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All the cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Wisent Inc., St Bruno, Qc, Canada) with 10% fetal bovine serum (HyClone Laboratories, Mississauga, ON, Canada), except for the A549 cell line which was cultured in DMEM supplemented with 5% fetal bovine serum.

Plasmids:

The plasmid pRK5 containing the myc-RasV12 gene with a glycine to valine mutation at amino acid position 12 and a sequence (EQKLISEEDLGS) containing a myc epitope inserted between a methionine and a threonine (amino acid positions one and two) was a generous gift from
Nathalie Lamarche-Vane, McGill University, Montreal, Canada. The plasmid was digested with the restriction enzyme *ClaI* and the 5’ overhang filled in with Klenow. The myc-RasV12 fragment was subsequently released by *ApaI*, and cloned into the *EcoRV/ApaI* site of *pIND*.

*pAN130* containing Raf-1 (20) served as a template to amplify the carboxyterminal, catalytic domain of Raf-1 (Raf-CT) using the oligos O-1 and O-2 listed in Table 1. At the same time an *EcoRI* site (printed in bold) was created to facilitate the cloning of the amplified 962 bp fragment into the *EcoRI/XhoI* sites of *pcDNA3*. A Kozak sequence (printed in italic) was inserted in frame by cloning the phosphorylated, annealed oligos O-3 and O-4 (Table 1) into the *EcoRI* site of Raf-CT, creating an *AflII* site (printed in bold). The nucleotide sequence was verified by sequencing. The Kozak-Raf-CT fragment was released by *AflII/XhoI* and cloned in the corresponding sites of *pIND*.

MEK-1 was released from *pAN104* (20) by *BamHI/XhoI* restriction digest and cloned into the corresponding sites of Bluescript KS. MEK-1SD was created by site directed mutagenesis (QuickChange™ Site-Directed Mutagenesis Kit, Stratagene) using two complementary oligos. The nucleotides that were changed in order to obtain a serine to aspartic acid mutation in MEK-1SD are underlined in O-5 in Table 1. The nucleotide sequence was verified by sequencing and the MEK-1SD was released from Bluescript with *BamHI/XhoI* and cloned in the corresponding sites of *pIND*.

The plasmid mycCMV5-ERK2-MEK1-LA was kindly provided by Melanie H. Cobb, University of Texas Southwestern, Dallas, USA. The plasmid was digested with *HindIII*, the 5’ overhang was filled in with Klenow fragment and ERK2-MEK1-LA was subsequently released by *KpnI* and cloned into the *KpnI/EcoRV* sites of *pIND*.

**Amplification of the hTFPI-2 promoter region:**

Genomic DNA was isolated from 293 cells. The cells were washed in PBS, lysed in a buffer containing 10mM NaCl, 10mM EDTA, 0.5% Sarkosyl and 10mM Tris pH=8 and incubated
with proteinase K (10mg/ml) at 50°C over night. After two phenol and two chloroform extractions, the genomic DNA was ethanol precipitated and dissolved in TE pH=8. A 1.5 kb fragment of the 5’-flanking region of the hTFPI-2 was amplified with an Expand High Fidelity PCR System (Boehringer Mannheim) using the oligos O-7 and O-8 (Table 1) to create a KpnI and a BglII restriction site (printed in bold). This KpnI/BglII fragment was cloned into the plasmid pXP2, which contains the firefly luciferase reporter (21) and which was a generous gift from Mark Featherstone, McGill University, Montreal, Canada. The resulting construct was named p–1511-luc. The sequence was found identical to the one published by Kamei (22) except for a C to A change at nucleotide position –47 relative to the translation start site.

Promoter Deletion Constructs:

The hTFP-2 promoter/luciferase reporter plasmids p-1293-luc, p-1055-luc, p-881-luc, p-733-luc, p-384-luc, p-222-luc, p-89-luc were created by restriction digest on the original p-1511-luc plasmid. The p-1511-luc plasmid was digested with Drai, Earl, Narl, MscI, EcoNI, EcoRI, and Smal, respectively, and the DNA fragments were released by BglII digest. The 5’-overhang created by Earl, Narl, EcoNI, EcoRI restriction digest were filled in with Klenow fragment and the restriction fragments cloned into the Smal/BglII site of pXP2.

Mutagenesis of single potential transcription binding sites:

The putative Sp1 transcription factor binding site GGGCGGG between nucleotide positions –190 and –184 was changed to GGGCGAA, the putative AP-1 site TGAATCA between nucleotide positions –162 and –156 was altered to GCTAGCA and the overlapping Sp1/AP-2 and GC box GGCTCCGCCCCGGCGGGG between nucleotide positions –144 and –126 was modified to GGCTTTGGCCCCAAGCGGG. Double stranded oligos containing the corresponding nucleotide changes were phosphorylated, annealed and cloned as HindIII/EagI fragments into the corresponding sites of p-1055-luc. Each mutation was confirmed by DNA sequencing. The oligos used for p-198MSP1A-luc were O-9 and O-10, for p-198MAP1-luc O-11 and O-12 and for p-
198MSP1B/MAP2-luc O-13 and O-14 (Table 1). p-222MAP1/MSP1B-luc and p-222MAP1/MAP2-luc were created by PCR using the Expand High Fidelity PCR System using the oligos O-15 and O-16 or O-17, respectively. In both constructs the putative AP-1 site TGAATCA between nucleotide positions –162 and –156 was altered to GCTAGCA and either the putative Sp1 site between nucleotide positions –140 and –134 CCGCCCC altered to TTGCCCCC or the putative AP-2 site between nucleotid positions –136 and –126 CCCGGCGGGGG altered to CCCGGCAGG. The sequence for the HindIII and EagI restriction sites are printed in bold and the nucleotide changes are underlined (Table 1). The PCR products were verified by sequencing and subcloned as HindIII/EagI fragments into the corresponding sites of p-1055-luc.

**Transient transfection:**

For inhibitor studies, the HEK293 cells (150000 cells/well) were seeded the day prior to transfection. The cells were cotransfected with 1μg of p-1511-luc and 70ng of a GFP-spectrin control plasmid (23). 24h after transfection, the cells were serum starved for 24h. 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), centrifuged at 12k for 5 min and the cell extract assayed for firefly luciferase activity using the luciferase reporter assay system (Promega). Light intensity was measured by using a microtitre plate luminometer (DYNEX Technologies, Inc, Chantilly, VA, USA). The GFP control vector allowed to correct for transfection efficiency. The cells in the second tube were trypsinized, washed and the percentage of cells that express GFP determined by cytelfluorometry (EPICS XL-MCL). Determination of protein by the Bradford assay (Bio-Rad) controlled for harvesting efficiencies. Luciferase activity was expressed as firefly light units/μg protein and normalized for transfection efficiency.

HEK293, HeLa, A549 and MCF7 cells were seeded into 6-well plates at 150000, 250000, 250000 and 400000 cells/well, respectively, one day prior to transfection. All cells were transfected with 1μg of the reporter gene constructs. HEK 293 and HeLa cells were transfected with Fugene at
a 3l FuGENE6/1g DNA ratio and A549 and MCF7 cells were transfected with Lipofectamine Plus according to the manufacture’s protocol. 24h after transfection, the cells of each well were split into 6 wells of a 24 well plate. 7h later, the cells were serum starved over night and subsequently treated with PMA 250nM or EGF 50ng/ml as indicated. Cell extracts were lysed and analyzed as described above. The fold stimulation of luciferase was calculated as firefly light units/g protein of PMA or EGF treated cells divided by the firefly light units/g protein of non treated cells.

Two days prior to transfection, EcR-293 cells cultivated in DMEM supplemented with 10% FBS and Zeocin (400\text{mg/ml}) were seeded into 6-well plates at 200000 cells/well. EcR-293 cells stably express the modified ecdysone receptor. A gene of interest, cloned into a pIND-based inducible expression vector, can be induced with the ecdysone analog pronasterone A that binds to the ecdysone receptor. Cells were transfected with 6l FuGENE6 and 1.07mg of total DNA (0.9 mg RasV12, RafCT, MEK-1SD, ERK2-MEK1-LA or pIND control vector, 0.1 mg reporter plasmid and 70ng GFP control plasmid). The medium containing FBS was replaced 24h after transfection with DMEM without serum, pronasteron A was added 4h later to the medium to induce protein expression at a concentration of 6\text{mM} unless indicated differently and cells were harvested 20h later. Luciferase activity was expressed as firefly light units/g protein and normalized for transfection efficiency.

**Western Blots:**

Cells were harvested and washed with PBS. The cells were lysed in PBS containing 0.1mM sodium vanadate, 10mM sodium pyrophosphate, 1.5% triton and the proteinase inhibitors aprotonin 10\text{mg/ml}, leupeptin 10\text{mg/ml} and 1mM PMSF. 15\text{mg} of total protein was separated by SDS-polyacrylamid electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 1% BSA in TBST (150mM NaCl, 0.05% Tween 20, 20mM TrisCl pH=7.5) and subsequently incubated with the anti-myc (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 1h at room temperature. The Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody was diluted 1:1000 in TBST containing
4% skin milk powder and incubated for 1h. After incubation with the secondary antibodies conjugated to horseradish peroxidase (1:1000) for 1h, the bands were visualized by ECL (Roche Diagnostics Corp., Indianapolis, IN, USA).
Results:

We have observed that hTFPI-2 was highly upregulated on microarrays that were probed with cDNAs originating from HEK293 cells treated with 250nM PMA for 4h (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 upregulated in BeWo and JEG-3 trophoblast cells treated with PMA (24).

To study the signaling pathway which 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 1511bp fragment of the 5’-flanking region of the hTFPI-2 from genomic DNA isolated from HEK293 cells and cloned the 1511bp 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 250nM PMA showed a 10-fold stimulation of luciferase compared to cells without PMA treatment (Figure 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 \( \mu \)M. UO126 completely inhibited PMA induction at a concentration of 100\( \mu \)M, suggesting that PMA activates the hTFPI-2 promoter through the MEK signaling pathway (Figure 1A).

As shown in the Western Blot in Figure 1B, PMA activated p44/p42 MAP kinase 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 observed in HEK293 cells was unique to this cell line or whether PMA promotes upregulation of the hTFPI-2 in other epithelial cells 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, while PMA induced the hTFPI-2 promoter activity in MCF7 and HeLa cells 30 or 20 fold, respectively (Figure 2). Since PMA has been reported to transactivate the epidermal growth factor receptor (26), we tested whether hTFPI-2 could be induced by the growth factor EGF. While EGF upregulated 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 (Figure 2).

Ras, Raf, MEK, and ERK can induce the hTFPI-2 promoter activity

To assess the importance of the components of the ERK/MAP kinase 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 activated signaling components, such as RasV12, RafCT, MEK-1SD, ERK2-MEK1-LA or the empty control vector (pIND). 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 (Figure 3A). Protein expression of RasV12, RafCT, MEK-1SD and ERK2-MEK1-LA in transiently transfected EcR-293 cells is shown on Western Blots in Figure 3B. All the constitutively activated signaling components were well expressed. Expression of RasV12 stimulated the luciferase reporter gene 37 fold compared to the control vector. Expression
of constitutively active signaling MAP-kinase components further downstream of Ras, such as RafCT, MEK-1SD and ERK2-MEK1-LA induced the luciferase reporter gene 7, 92 or 39 fold, respectively (Figure 3A), indicating that hTFPI-2 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), while the RasV12 and ERK2-MEK1-LA fusion protein (28) activated the hTFPI-2 promoter to a similar extent. In conclusion, these results demonstrate the Ras/Raf/MEK/ERK signaling pathway mediates regulation of the hTFPI-2.

The minimal inducible promoter activity of hTFPI-2 is located between the –89/-222bp 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 pIND. Fold stimulations of the Ras versus control vector is shown in Figure 4A. 35 to 51 fold stimulation of luciferase was obtained with constructs p-1511-luc through p-222-luc, while no inducible luciferase activity was obtained with construct p-89-luc, suggesting that the minimal inducible promoter activity is located between nucleotide positions –222 and –89 (Figure 4A). Similar results were obtained in PMA treated HEK293 cells expressing the deletion constructs. PMA stimulated p-1511-luc through p-222-luc 5 to 8 fold, while p-89-luc was not induced by PMA (Figure 4B). Therefore, we decided to investigate the promoter elements in the –222/-89 region that are responsible for the 51 fold Ras and 8 fold PMA stimulation as compared to control cells.

The minimal basal promoter activity includes the –89/-384bp region, since p-384-luc showed similar basal luciferase activity as longer constructs, while no basal activity was obtained with construct p-89-luc (Table 2). As shown in Table 2, the luciferase activity dropped by 74% in
vector (pIND) transfected cells expressing p-222-luc compared to cells expressing p-384-luc, indicating that the –384 to –222bp region 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 384bp of an upstream promoter segment compared to cells transfected with RasV12 and a reporter construct containing only a 222bp promoter segment (Table 2). 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 can not rule out that the –384/–222bp region may contain additional enhancer elements that can contribute to the inducible activity.

INSERT TABLE 2 HERE

Identification of an AP-1 site as specific inducible DNA response elements

To determine potential cis-acting elements responsible for the PMA and Ras inducibility of the -89/-222bp promoter region, we used the computer program TFSEARCH ver.1.3 that searches highly correlated sequence fragments versus the TFMATRIX transcription factor binding site profile database by E. Wingender, R. Knueppel, P. Dietze, H. Karas (GBF-Braunschweig). Several putative transcription factor binding sites were identified, including a potential Sp1 site (CGGCGCGCGCGG) between nucleotide positions –192 and –179, a potential AP-1 site (ATGAAATCA) between positions –163 and –156 and an overlapping Sp1 (underlined)/AP-2 and GC box (GGCTCCCCGGCGGGG) between positions –144 and –126 (Figure 5).

INSERT FIGURE 5 HERE

To assess the importance of these sites, we mutated the presumptive Sp1 site (GG at position –185/–184 mutated to AA, printed in bold above), mutated the presumptive AP-1 (TGAAT at positions –162 to –158 mutated to GCTAG) or altered the presumptive Sp1 and AP-2 site (CC at positions –140/–139 mutated to TT and GG at positions –133/–132 mutated to AA; printed in bold above). The resulting reporter gene plasmids p-198MSP1A-luc, p-198MAP1-luc, 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 Figure 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 to p-222-luc. The reporter construct p-198MSP1A was still 52 fold induced by RasV12 as compared to the control vector (Table 2). The loss of some basal activity might be due to the mutation of the consensus Sp1A site or the absence of the –198/222 region. Mutation of the consensus AP-1 site caused a considerable decrease in inducible activity, while substantial basal activity was still retained, indicating that this putative AP-1 site is important for inducible activity. Furthermore, mutation of the overlapping consensus Sp1B/AP-2 site affected basal and inducible activity severely (Figure 6). Deletion of the consensus –156 AP-1 and the consensus –126 AP-2 site caused only a minor decrease in inducible activity as compared to p-198MAP1-luc, a construct that had only the consensus –156 AP-1 site mutated, suggesting that the putative –126 AP-2 site may have a subtle effect on inducible promoter activity. Substantial basal activity, however, was still retained comparable to p-198MAP1-luc (Figure 6). Deletion of the consensus –156 AP-1 and the consensus –134 Sp1 sites in the –222bp 5’flanking region of the 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 (Figure 6). Similar results were obtained with another MAP kinase 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.

INSERT FIGURE 6 HERE
Discussion:

A schematic summary of the results presented in this study is shown in Figure 7. We showed that \textit{hTFPI-2} is upregulated in several epithelial cells following stimulation by PMA (Figure 2). Additionally, EGF, a growth factor, was able to stimulate promoter activity of \textit{hTFPI-2} as shown in HeLa cells (Figure 2). In HEK 293 cells, induction of the promoter activity by PMA could be blocked by the MEK specific inhibitor UO 126 (Figure 1), suggesting that PMA induction of \textit{hTFPI-2} is mediated through a pathway that involves MEK. Indeed, activated Ras, Raf, MEK, and ERK were able to promote gene transcription (Figure 3), indicating that the Ras/Raf/MEK/ERK signaling pathway is necessary for promoter activation. The ERK/MAP kinase pathway activates members of the Fos gene family which dimerize with Jun family members and bind as the AP-1 complex to a consensus DNA sequence 5'-TGAG/CTCA-3' (29). We located an AP-1 consensus site at position –156 to –162 as a \textit{cis}–acting element essential for inducible promoter activity. Additionally, a Sp1 consensus site at position –134 to –140 of the \textit{hTFPI-2} promoter was essential for basal promoter activity (Figure 6).

Our study in HEK 293 cells indicates that \textit{hTFPI-2} 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 \textit{hTFPI-2}. In agreement with these reporter gene studies, we observed upregulation of the \textit{hTFPI-2} in preliminary microarray experiments comparing RasV12, RafCT, MEK-1SD or ERK2-MEK1-LA expressing EcR-293 cells to non-transfected cells (data not shown). RasV12 is a strong activator of the Raf/MEK/ERK pathway, since it contains a glycine to valine mutation at amino acid position 12 and therefore remains in the active GTP-bound state (30). In contrast, RafCT, the carboxy-terminal part of Raf that lacks the regulatory amino-terminal domain is a moderate activator of the MEK/ERK pathway (31). Consistent with this, we observed a 37 fold induction of \textit{hTFPI-2} by Ras and a 7 fold induction by RafCT (Figure 3). Similarly, the stronger activator Raf-CAAX, a full length Raf containing a CAAX-box (32) induces higher (15 fold) induction of \textit{hTFPI-2} promoter activity than RafCT (data not shown).
The tumor promoting agent PMA often serves as a model agent to study the mechanism by which growth factors regulate growth and differentiation of the cells. PMA mimics the action of diacylglycerol, the endogenous activator of protein kinase C (PKC) (33,34). PKC activates Raf by direct phosphorylation (35). However, activation of Raf by PKC is Ras-dependent in several cell lines (36,37). Not all the phorbol ester responses can be explained by the PKC action and some responses may be attributed to non kinase phorbol ester receptors such as the \(-\) and \(-\)-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 MAP kinases, such as the mitogen-responsive ERKs (extracellular signal regulated kinases), the stress-responsive JNK/SAPKs (c-Jun N-terminal kinase/stress activated protein kinases) and p38 MAP kinases. The pathways induced by PMA are cell line dependent; for example PMA activates the JNK MAP kinase pathway in normal oral keratinocytes, but not in immortalized/transformed keratinocytes, HeLa cells or HEK293 cells (39,40). In this paper we provide evidence that \(hTFPI-2\) expression 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 \(hTFPI-2\) promoter activity by PMA. Since UO126 inhibited PMA promoter activation at the commonly used concentration of 10\(\mu\)M, we hypothesize that the ERK/MAP kinase pathway may be sufficient for PMA induction of \(hTFPI-2\). Besides affecting the PMA dependent \(hTFPI-2\) promoter activation, UO126 had a minor negative effect on basal levels of \(hTFPI-2\) transcription, probably due to the low level of activation of \(hTFPI-2\) by endogenous MAP kinase signaling components.

Activation of the ERK/MAP kinase causes induction of \(fos\) genes through phosphorylation of ternary complex factors (41). Indeed, we have observed upregulation of \(fosB\) when PMA-induced HEK293 cells were probed on microarrays (data not shown). Fos heterodimerizes with Jun to form the AP-1 complex that 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 –384 to –89bp region of the *hTFPI-2* promoter consistent with a previous promoter deletion study in human transformed bone marrow microvascular endothelial cells (22), identifying a 85bp fragment (corresponding to the –299 to –214bp 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 –222 to –89bp promoter region.

The –222 to –89bp region is sufficient for a 52 fold Ras and an 8 fold PMA induction of the *hTFPI-2* promoter activity as compared to basal level (Figure 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 (Figure 6).

Recently, Konduri (19) identified a 231bp 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, since we did not find such elements in HEK293 cells. Similar luciferase activities were observed in constructs of various lengths between 1511 and 384 induced by Ras (Table 2) or PMA (data not shown). Konduri suggested that three potential AP-1 sites (corresponding to -309 to –299; -213 to –203; and –162 to –156 in our study) may be involved in PMA related gene induction (19). While we identified the –156 to –162 consensus AP-1 site as essential for transcription of *hTFPI-2*, our results suggest that the –213 to –203 promoter segment may not be essential for Ras related gene induction in EcR-293 cells, since the promoter segment of construct p-198MSP1A-luc, that lacks the –213/–203 segment, is sufficient for a 52 fold Ras induction similar to longer constructs (Table 2).

The ERK/MAP kinase pathway stimulated by growth factors and phobol esters is associated with cellular proliferation and differentiation. The biological consequences of hTFPI-2
induction by agents stimulating the ERK/MAP kinase pathway and the role of hTFPI-2 in tumor progression is not fully understood. On one hand, several cell lines stably overexpressing hTFPI-2, such glioma SNB19 cells, choriocarcinoma JAR cells, amelanotic melanoma cells and prostate cancer cells were less invasive than their parental cells (14 -17) and addition of recombinant hTFPI-2 inhibited the invasiveness of SNB19 glioma cells as measured in a matrigel assay (42). On the other hand, hTFPI-2 has been shown to have a proinvasive effect in cancer. Recombinant hTFPI-2 has been shown to be a mitogen for vascular smooth muscle (43) and hTFPI-2 potentiates HGF-induced invasion of HCC cells and is capable of inducing invasion of HCC on its own (18). Bimodal function has also been described for tissue inhibitors of metalloproteinases, another family of tissue inhibitors. Tissue inhibitors of metalloproteinases inhibit the activity of matrix metalloproteinases by binding to their zinc binding site, but they are also involved in cell surface-targeted MMP activation cascade, they stimulate growth and are antiangiogenic (44). Similarly, the TFPIs may also perform a very wide variety of biological activities and transcriptional regulation of TFPIs may play an important role in the balance of protease activities resulting in the metastatic/invasive phenotype.

**Acknowledgements:**

The authors would like to thank Dr. M. Cobb for the generous gift of the plasmid mycCMV5-MEK1-Erg2-LA, Dr. N. Lamarche-Vane for the plasmid pRK5 myc-RasV12 and Dr. M. Featherstone for the luciferase containing vector pXp2. We also would like to thank Dr. M. Featherstone and Dr. M. Jaramillo for constructive comments on this manuscript. This work was supported by the NRC GHI program. This is NRCC publication number XXXXX.
References:


Footnotes:
The abbreviations used are: AP-1, activating protein–1; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; FCS, fetal calf serum; hTFPI-2, human tissue factor inhibitor-2; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline; PMA, Phorbol 12-Myristate 13-acetate, PKC, protein kinase C
Figure Legends:

Figure 1:

**Induction of the hTFPI-2 promoter activity by PMA is inhibited by the MEK inhibitor UO126:** A) HEK 293 cells were transiently cotransfected with the luciferase reporter plasmid p-1511-luc and a control vector containing GFP. 24h later, the cells were serum starved for 24h. UO126 was added at a concentration of 10 or 100µM 15min prior to 250nM PMA treatment for 4h. Luciferase activity was calculated as light units/mg protein and normalized for transfection efficiency. Data are shown as mean +/- SD from one representative experiment performed in duplicate transfections. B) Inhibition of Phospho-p44/42 MAPKinase by UO126. HEK293 cells were incubated with 250nM PMA for 4h (lane 1), and treated with 0.1, 1 or 10µM UO126 prior to PMA treatment (lanes 2, 3, and 4). Proteins were separated on a 12% SDS-polyacrylamid gel and subjected to Western blotting with the Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody.

Figure 2:

**Stimulation of hTFPI-2 promoter activity by PMA and EGF in various epithelial carcinoma cell lines:** A549, MCF7 and HeLa cells were transiently transfected with p-1511-luc. The cells were serum starved for 16h and subsequently treated with 250nM PMA or 50ng/ml EGF for 6h. The fold stimulation of luciferase was calculated as firefly light units/mg protein of PMA or EGF treated cells divided by the firefly light units/mg protein of non treated cells. Data are shown as mean +/- SD from at least three independent transfection experiments performed in duplicates.

Figure 3:

**Induction of the hTFPI-2 promoter activity by RasV12, RafCT, MEK-1SD and ERK2-MEK1-LA:** A) EcR-293 cells were cotransfected with RasV12, RafCT, MEK-1SD, ERK2-MEK1-LA or pIND control vector and reporter plasmid p-1511-luc. A vector containing GFP was included to control for transfection efficiencies. The cells were serum starved 4h prior to induction of protein
expression with pronasteron A for 20h. The fold stimulation of luciferase was calculated as normalized firefly light units/µg protein of cells expressing signaling components divided by the normalized firefly light units/µg protein of cells transfected with the control vector pIND. Data are shown as mean +/- SD from one representative experiment performed in duplicate transfections. B) EcR-293 cells were transfected with 1µg pIND (lanes1, 3 and 5) RasV12 (lane 2), RafCT (lane 4), MEK-1SD (line 6) or ERK2-MEK1-LA (line 7). 24h later, the cells were serum starved 4h prior to induction of protein expression with pronasteron A at a concentration of 10µM for 20h. Proteins were separated on 15% (lanes 1-4) or 12% (lanes 5-7) SDS-polyacrylamid gels and subjected to Western blotting with anti-myc (9E10) (lanes 1 and 2), anti-Raf-1 (C12) (lanes 3 and 4), or anti-MEK1 (C18) (lanes 5-7).

Figure 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 4h prior to induction of protein expression with pronasteron A for 20h. The fold stimulation of luciferase was calculated as normalized firefly light units/µg protein of cells expressing signaling components divided by the normalized firefly light units/µg protein of cells transfected with the control vector pIND. Data are shown as mean +/- SD from at least three independent transfection experiments. B) HEK293 cells were transiently transfected with the deletion constructs. The cells were serum starved for 16h prior to treatment with 250nM PMA for 4h. The fold stimulation of luciferase was calculated as firefly light units/µg protein of PMA treated cells divided by the firefly light units/µg protein of mock treated cells. Data are shown as mean +/- SD from at least three independent experiments performed in triplicates.
Figure 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 bold and the position indicated by numbers.

Figure 6:
Mutagenesis of potential transcription factor binding sites: EcR-293 cells were transiently cotransfected with RasV12 or control vector pIND and luciferase reporter gene constructs containing modified potential transcription factor binding sites. Cotransfection with a vector containing GFP controlled for transfection efficiency. The cells were serum starved 4h prior to induction of protein expression with pronasteron A for 20h. Luciferase activity was calculated as light units/mg protein and normalized for transfection efficiency. Data are shown as mean +/- SD from at least four independent transfection experiments.

Figure 7:
Schematic summary of MAP kinase pathway that regulates the activity of the hTFPI-2 promoter: The MAP kinase signaling components involved in activation of hTFPI-2 are indicated in bold and cis–acting element essential for inducible promoter activity are indicated in boxes.
### Table 1: Oligonucleotides

<table>
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<tr>
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<th>Sequence</th>
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<td>5’-GGGAATTCAGGCCCTCGTGACGACAGAGATTCAAGC-3’</td>
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<tr>
<td>2</td>
<td>5’-GTAACTCGAGTCAACTAGAACAGAGGAGCC-3’</td>
</tr>
<tr>
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Table 2: Luciferase activity of the deletion constructs described in Figure 4.

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Figure 1:

(A) Normalized Luciferase Activity (RLU/µg protein)

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<th>10 µM</th>
<th>100 µM</th>
<th>100 µM</th>
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<td>no</td>
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(B) Western Blot

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<th>(µM)</th>
</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
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</table>
Figure 2:

![Bar chart showing fold stimulation of luciferase](chart.png)
Figure 3:

A

Fold Stimulation of Luciferase

RasV12  RafCT  MEK-1SD  ERK2-MEK1-LA

B

RasV12

RafCT

ERK2-MEK1-LA
Figure 4: AB

B

A

PMA

RAS

TFPI-2 Promoter Sequences

Fold Stimulation of Luciferase

p-1511-luc
p-1293-luc
p-1055-luc
p-881-luc
p-733-luc
p-384-luc
p-222-luc
p-89-luc

minimal inducible promoter activity
Figure 5:

(-222) AATTCCTCTCCCTCTTACACAGTTTGCAGCGC

GGGGGC

GG

CGGGGTGACAGTCCCCGTGCA TGA ATCA

TGA

A

T

CA

GCCACCCCTCAGGCT

CC

GCC

GG

GGGTCGGCC

GGACGCTCGCCCCGCATAAAGCGGGCACCC

Sp1

GC box

AP-2

Sp1

-162 to-158

-185/184

-140/139

-133/132

-130/129

-130/129
Figure 6:

- **p-198MSP1A-luc**: SP1A
- **p-198MAP1-luc**: AP1
- **p-198MSP1B/MAP2-luc**: SP1B/AP2
- **p-222MAP1/MAP2-luc**: AP1, AP2
- **p-222MAP1/MSP1B-luc**: AP1, SP1B

**pIND Basal Activity**

- **25**
- **50**
- **75**

**RAS Inducible Activity**

- **25**
- **50**
- **75**

% Luciferase Activity of p-222-luc
Figure 7:

```
Figure 7:

Receptor tyrosine kinase
  GRB2 mSOS
  Ras
  Raf
  MEK1/2
  ERK1/2

PMA

nucleus

Jun Fos

AP-1 Sp1
-156 -134

"ON"

RasV12
RafCT
MEK-1SD
ERK2-MEK1-LA

UO 126

TFPI-2
```
The ERK/MAP kinase pathway regulates the activity of the human tissue factor pathway inhibitor-2 (hTFPI-2) promoter
Christina Kast, Minglun Wang and Malcolm Whiteway

J. Biol. Chem. published online November 24, 2002

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