Regulation of Tissue Factor Gene Expression in Epithelial Cells
Induction by Serum and Phorbol 12-Myristate 13-Acetate

Mei-Zhen Cui, Graham C.N. Parry, Thomas S. Edgington, Nigel Mackman

Abstract

Cell-specific expression of tissue factor (TF) in vivo is consistent with its primary role in hemostasis. In addition, TF expression is induced in cultured cells by a variety of agents, including serum and growth factors, which define the TF gene as a "primary response" gene. In this study we examined the signaling pathways and cis-acting regulatory elements required for induction of TF gene expression in HeLa cells in response to serum and the tumor promoter, phorbol 12-myristate 13-acetate (PMA). TF activity and mRNA were induced greater than sixfold in quiescent HeLa cells by serum and PMA. TF mRNA induction by both agonists required intracellular Ca\(^{2+}\) mobilization, whereas inhibition of protein kinase C abolished induction of the TF gene by PMA but had no effect on induction by serum. Functional studies demonstrated that a region of the human TF promoter between \(-96\) and \(+121\) bp contained regulatory elements required for serum and PMA induction. These data indicate that different signaling pathways regulate TF gene expression in response to serum and PMA, although the same cis-acting DNA elements may mediate induction. (Arterioscler Thromb. 1994;14:807-814.)

Key Words • tissue factor • serum induction • gene expression

The distribution of human tissue factor (TF) in vivo as an envelope around blood vessels, organ capsules, and epithelial surfaces is consistent with its significant role as a hemostatic barrier to prevent excessive bleeding in the event of vascular injury.\(^1\)\(^2\) For instance, adventitial fibroblasts surrounding blood vessels constitutively express high levels of TF. In contrast, this protein is not normally expressed by intravascular cells, although during disease processes such as atherosclerosis, septic shock, and various forms of cancer, monocytes/macrophages and endothelial cells can be induced to express TF and thereby initiate various forms of intravascular coagulation.\(^3\)\(^5\)

In vitro studies indicate that TF gene expression is highly regulated and can be induced in monocytes/macrophages and endothelial cells by many agents, including bacterial lipopolysaccharide (LPS), the inflammatory cytokines interleukin-1 and tumor necrosis factor-\(\alpha\), phorbol esters, and oxidized low-density lipoprotein.\(^6\)\(^11\) In addition, TF expression is induced by serum and growth factors that include fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, and transforming growth factor type \(\beta\).\(^12\)\(^-17\) This led to the definition of the TF gene as a "primary response" or "immediate early" gene because it is induced in the absence of de novo protein synthesis.\(^14\)\(^16\) It is noteworthy that induction of the TF gene exhibits kinetics similar to that of \(c-myc\), with mRNA levels peaking at 2 hours. This response is delayed when compared with the induction of other primary response genes such as \(c-fos\).\(^14\) Taken together, this complex pattern of regulation suggests that TF may play a role in processes other than blood coagulation, such as cell proliferation in response to vascular injury and in inflammation.\(^16\)\(^18\)

Isolation of the complete human TF gene has revealed that the promoter is located within a CpG island that contains four putative Sp1 binding sites.\(^19\) Functional analysis of the TF promoter in our laboratory localized a region between \(-111\) and \(+14\) (relative to the transcription start site) that was required for serum induction.\(^13\) Importantly, this region, as for the 5' flanking region of \(c-myc\), did not contain DNA sequences resembling the serum response element that has been defined in the c-fos promoter,\(^20\) suggesting an alternative mechanism of serum induction.

The present study investigated signaling pathways and cis-acting DNA elements that regulated induction of TF gene expression in quiescent epithelial cells exposed to serum or phorbol 12-myristate 13-acetate (PMA). Our results demonstrated that induction of TF mRNA by both agonists was dependent on Ca\(^{2+}\) mobilization but that only PMA induction required activation of protein kinase C (PKC). Deletional analysis of the TF promoter revealed that a region between \(-96\) and \(+121\) bp retained cis-acting regulatory elements required for serum and PMA induction.

Methods

Phorbol 12,13-dibutyrate (PDBu), PMA, and cycloheximide (CHX) were obtained from Sigma Chemical Co; Bisindolylmaleimide GF 109203X (GF 109203X), quin 2-AM, and the calcium ionophore A23187 were obtained from Calbiochem.

Cell Culture and Plasmids

The HeLa cell line was obtained from the American Type Culture Collection and maintained in Dulbecco's modified
Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were serum-starved by culturing in DMEM plus 0.5% FBS for 48 hours before induction with 20% FBS. Plasmids containing the Rous sarcoma virus (RSV) promoter, pRSVLuc and pRSVCAT, and the promoterless p19LUC plasmid were used as controls in the transfections. The majority of TF promoter plasmids have been described previously. Site-directed mutagenesis of the putative EGR-1 and Sp1 sites within pTF(-111)LUC was performed as described. The EGR-1 site (underlined) at -74 bp, 5’-GGCGGGGGCG-3’, was mutated to 5’-TCGAGGGGCG-3’. The same EGR-1 site and overlapping Sp1 site (doubly underlined) at -70 bp, 5’-GCGGGGGGCGGGCGGCGG-3’, were mutated simultaneously to 5’-GCGAGAGTCGGACG-3’. A region of the TF promoter between -111 and -39 bp was cloned upstream of the minimal thymidine kinase (TK) promoter to create pTF(-111/-39)TKLUC. Ten micrograms of supercoiled plasmid DNA was used to transfect each subconfluent cell culture (10-cm plate) with DEAE-dextran. One-half microgram of pRSVCAT was used to control for variations in transfection efficiency. Transfected cells were serum-starved by culturing in DMEM plus 0.5% FBS for 48 hours before a 5-hour induction.

Activity Assays
Luciferase activity in cell lysates from transfected cells was determined with the luciferase assay system (Promega Corp). Chloramphenicol acetyl transferase (CAT) activity was measured as described previously. TF activity was assayed in cell lysates solubilized with 15 mmol/L octyl-β-D-glucopyranoside at 37°C for 15 minutes by using a one-stage clotting assay. A pool of neutralizing TF monoclonal antibodies (TF8-5G9, TF8-6B4, and TF9-9C3) demonstrated that the observed procoagulant activity was due to TF protein.

RNA Hybridization
Total cellular RNA was isolated and subjected to denaturing electrophoresis in formaldehyde/agarose gels. RNA was blotted onto Gene Screen (New England Nuclear) and hybridized with radiolabeled cDNA probes. A 641-bp human TF cDNA fragment was used to detect TF mRNA, whereas a 900-bp glucose 6-phosphate dehydrogenase (G6PDH) cDNA fragment was used to normalize RNA loadings.

Electrophoretic Mobility-Shift Assays (EMSA)
EMSAs were performed on the Gel Shift System (Promega Corp). The following double-stranded oligonucleotides were used: AP-1 consensus, 5’-CGGTTGATGAATCGGG-3’; TF distal AP-1 site, 5’-CGGGTTGATACTCGGGG-3’; and TF proximal AP-1 site, 5’-CGGGTTGAGTCATCCCTT-3’. Nuclear extracts were prepared as described previously.

Cell Viability
The viability of cells exposed to PKC antagonists or Ca2+ chelators was monitored by light microscopy, trypan blue uptake, and general protein synthesis ([35S]methionine incorporation). HeLa cells were radiolabeled with [35S]methionine (1036 Ci/mmol; Amersham Corp), and the cell lysates were precipitated with 10% trichloroacetic acid to assess radiolabel incorporation. Cell viability was 85% to 95% of controls in all experiments.

Results

Induction of TF Procoagulant Activity
Quiescent HeLa cells expressed a low basal level of TF activity that was rapidly increased by the addition of medium containing 20% serum (Fig 1a). Maximal levels of TF activity were observed 6 hours after induction, and TF activity remained elevated after 24 hours compared with the basal expression of quiescent HeLa cells. The induction of TF activity in response to serum was 9.5±2.6-fold (mean±SD, n=8). These results indicated that HeLa cells represented a suitable model to examine the intracellular mechanisms involved in serum induction of TF expression.

For comparison, we examined the regulation of TF expression in response to PMA. TF activity of quiescent HeLa cells was increased by PMA with kinetics similar to those observed with serum (Fig 1b). However, in contrast to serum stimulation, enhanced TF activity induced by PMA declined after 24 hours to the basal level observed in quiescent cells. The induction of TF activity in response to PMA was 6.4±0.6-fold (n=5). PMA concentrations between 10 and 50 ng/mL led to a dose-dependent induction of TF activity with maximal induction at 50 ng/mL (data not shown). Subsequent studies compared the similarities and differences between serum and PMA induction of TF gene expression.

Induction of TF mRNA
Induction of TF activity could be due to an increase in TF gene expression or an increase in the rate of initiation of coagulation by existing TF protein. Therefore, we determined whether changes in TF mRNA levels could account for the observed induction of TF activity. Quiescent HeLa cells contained a low steady-state level of TF mRNA that was rapidly induced by serum or PMA (Fig 2a and 2b). Maximal levels of TF mRNA were observed 6 hours after induction.
mRNA were observed 2 hours after serum stimulation, whereas induction by PMA was slightly delayed, reaching maximal levels between 2 and 5 hours. TF mRNA levels declined with both serum and PMA by 8 hours. The induction of TF mRNA was 11.0±1.8-fold (n=5) by serum and 13.3±3.9-fold (n=3) by PMA. These data indicated that serum and PMA increased the steady-state levels of TF mRNA in HeLa cells.

**Effect of CHX on TF mRNA Induction**

To determine whether serum and PMA induction of TF mRNA was dependent on preexisting cellular proteins or required de novo protein synthesis, quiescent HeLa cells were stimulated in the presence or absence of the translational inhibitor CHX. Serum and PMA-induced TF mRNA was not blocked by CHX (Fig 3a and 3b). In addition, CHX alone slightly increased TF mRNA levels and superinduced TF mRNA when coincubated with PMA. Thus, the induction mechanisms elicited by serum and PMA did not require de novo protein synthesis.

**Localization of cis-Acting Regulatory Elements in the TF Promoter**

Previous studies have demonstrated that serum stimulation of mouse fibroblasts increases the rate of TF gene transcription. Moreover, we have shown that a region of the human TF promoter between -111 and +14 is required for serum induction in monkey COS-7 cells. In the present study, human HeLa cells were used to examine serum induction of a more extensive 5′ deletion series of the human TF promoter to further localize cis-acting regulatory elements that mediated induction by serum. pTF(-278)LUC, containing a region of the TF promoter between -278 and +121, expressed a level of luciferase activity that was designated as 100% and was induced 5.8±0.3-fold (n=3) by serum (Fig 4). Deletion of a region between -278 and -97 reduced promoter activity but did not affect the magnitude of induction by serum; pTF(-111)LUC and pTF(-96)LUC were still induced 7.2±0.7-fold (n=6) and 5.6±0.2-fold (n=3), respectively. In contrast, deletion of an adjacent region between -96 and -68 to create pTF(-67)LUC abolished both detectable promoter activity and serum induction. pTF(-33)LUC and pTF(-21)LUC did not express any detectable promoter activity. These data indicated that a region of the TF promoter between -96 and +121 retained cis-acting DNA elements required for serum induction. These regulatory elements may overlap with DNA sequences required for basal promoter activity, because deletion of the region between -96 and -68 abolished both promoter activity and serum induction.

Similar transfection studies were performed to localize the PMA response element in the TF promoter. Because reports have indicated that AP-1 sites can mediate induction by PMA, initial studies determined the role of the two putative AP-1 sites within the TF promoter in PMA induction. To establish that these two sites could bind AP-1 in HeLa cells, EMSAs were performed with nuclear extracts from HeLa cells cultured in 10% FCS. The mobility of the protein-DNA complex formed with oligonucleotides containing the TF AP-1 sites, -223 distal (5′-TGAATCA-3′) (Fig 5, lane 7), was consistent with the binding of AP-1 to an oligonucleotide containing an AP-1 consensus site (Fig 5, lane 1). In addition, these complexes were specifically competed with an oligonucleotide containing an unlabeled AP-1 consensus site (Fig 5, lanes 5 and 8) but not with an oligonucleotide containing an unlabeled AP-2 site (Fig 5, lanes 6 and 9). The oligonucleotide containing the distal AP-1 site bound less protein than that...
containing the proximal AP-1 site, which may be attributable to the presence of a nonconsensus adenine nucleotide at position 4 of the distal site. Competition studies of AP-1 binding with an oligonucleotide containing an AP-1 consensus site confirmed that the proximal TF site exhibited greater affinity for AP-1 than the distal TF site (data not shown). EMSAs were also performed with nuclear extracts prepared from quiescent and PMA-stimulated HeLa cells. Quiescent HeLa cells exhibited constitutive AP-1 activity, as monitored by binding to oligonucleotides containing an AP-1 consensus site and the two TF AP-1 sites, that was increased by PMA (Fig 6). Taken together, these data suggest that the AP-1 sites in the TF promoter may mediate PMA induction.

To examine the functional role of these two AP-1 sites, we determined the level of PMA induction of the wild-type TF promoter and its derivatives containing mutations in the AP-1 sites. The wild-type TF promoter, pTF(−278)LUC, was induced 10.5±1.0-fold (n=4) by PMA. Unexpectedly, mutation of one or both of the AP-1 sites in pTF(−278mut1)LUC, pTF(−278mut2)LUC, and pTF(−278mut3)LUC did not significantly reduce PMA induction (Fig 4). Therefore, binding of AP-1 to these two sites did not appear to be required for PMA induction of TF gene expression. In fact, deletion of region −278 to −112, which contained the two AP-1 sites, did not reduce the magnitude of PMA induction because pTF(−111)LUC was still induced 8.5±3.3-fold (n=11) (Fig 4). In addition, pTF(−96)LUC expressed a similar high level of induction, 8.4±1.2-fold (n=3).

Plasmids pTF(−67)LUC, pTF(−33)LUC, and pTF(−21)LUC did not exhibit any promoter activity. These studies suggest that serum and PMA induction of the TF promoter may be mediated by adjacent or identical cis-acting regulatory elements that are proximal to position −96. One possible candidate was an overlapping EGR-1/Spl site at −70 (Fig 4). A similar element appeared to function as both a positive and negative regulator of the mouse adenosine deaminase promoter.35 To examine the role of these sites in serum and PMA induction, the EGR-1 site was mutated either alone or simultaneously with the Spl site (see “Methods”). The level of serum and PMA induction directed by pTF(−111mut1)LUC and pTF(−111mut2)LUC was not significantly reduced compared with the wild-type promoter (Fig 4). As an alternative approach to identify regulatory elements, a region between −111 and −39 of the TF promoter was cloned upstream from the minimal TK promoter to create pTF(−111/−39)TKLUC. However, this region of the TF promoter alone was unable to confer serum or PMA inducibility to the TK promoter (data not shown). Therefore, during this study we could not identify discrete nucleotide sequences in the TF promoter that mediated serum or PMA induction. Nevertheless, these data did reveal that a region of the TF promoter between −96 and +121 was required for both serum and PMA induction in HeLa cells.

![Graph showing serum and PMA induction of plasmids (p) containing the human TF promoter.](http://atvb.ahajournals.org/)

Fig 4. Graphic showing serum and PMA induction of plasmids (p) containing the human TF promoter. The position of putative binding sites for AP-1, Sp-1, EGR-1, AP-2, and late SV40 factor (LSF) are indicated, together with the serum response region between −111 and +14 bp. Serum and PMA induction of six plasmids containing 5' truncations of the TF promoter between −278 and −21 bp were examined. In addition, plasmids containing mutations in the AP-1 sites (−278mut1 to mut3) and the overlapping EGR-1/Spl site (−111mut1 and mut2) were induced. Bent arrow represents the start site of transcription of the TF gene, and the black box indicates the 5' untranslated region of the cloned TF gene upstream from the luciferase reporter gene (white box). Total luciferase activity (light units) from transfected HeLa cells was measured and normalized for the amount of DNA uptake with pRSVCAT. p19LUC and pRSVLUC were used as controls to indicate relative levels of promoter activity. Average n-fold induction of luciferase activity expressed by each plasmid in response to serum or PMA is shown ± SD; the number in parentheses indicates the number of independent experiments. NT indicates conditions that were not tested; RSV, Rous sarcoma virus.
Fig 5. EMSA showing nuclear protein binding to distal and proximal AP-1 sites of the tissue factor (TF) promoter. A nuclear extract was prepared from HeLa cells cultured in 10% fetal calf serum as described previously. Extracts were incubated with \(^{32}\)P-labeled double-stranded oligonucleotide probes containing either an AP-1 consensus site (lanes 1 through 3), the distal TF AP-1 site (lanes 4 through 6), or the proximal TF AP-1 site (lanes 7 through 9). The protein-DNA complexes were competed (Comp.) with a 10-fold molar excess of unlabeled oligonucleotides containing either an AP-1 site (lanes 2, 5, and 8) or an AP-2 site (lanes 3, 6, and 9) supplied in the Gel Shift System (Promega). Complexes were resolved on low-ionic-strength 4% nondeaturing polyacrylamide gels.

**Signaling Pathways Involved in TF mRNA Induction by Serum and PMA**

Inhibitors of various signaling pathways were used to investigate the intracellular mechanisms involved in induction of increased TF mRNA in HeLa cells. The Ca\(^{2+}\) mobilization signaling pathway was examined by using EGTA to chelate extracellular Ca\(^{2+}\) and quin 2-AM to chelate intracellular Ca\(^{2+}\) under established conditions. Reduction of extracellular Ca\(^{2+}\) by EGTA did not diminish induction of TF mRNA by serum or PMA, but chelation of intracellular Ca\(^{2+}\) with quin 2-AM alone (data not shown). These results suggested that Ca\(^{2+}\) mobilization was required for TF mRNA induction by both serum and PMA. Moreover, the Ca\(^{2+}\) ionophore A23187 induced TF mRNA to levels that were comparable with those observed in serum-induced cells (Fig 7), again indicating that TF mRNA expression was regulated by intracellular Ca\(^{2+}\) mobilization.

PMA is a direct activator of PKC that has been shown to be important in the induction of a number of primary response genes such as c-fos and c-myc. Therefore, we examined the role of the PKC pathway in the induction of TF mRNA. First we used the potent and specific PKC inhibitor bisindolylmaleimide GF 109203X as described. Pretreatment of quiescent cells for 30 minutes with GF 109203X completely abolished induction of TF mRNA by PMA (Fig 8). In contrast, this PKC inhibitor did not reduce serum induction of TF mRNA (Fig 8). Second, to independently examine the role of PKC in the induction of TF mRNA, PKC activity was downregulated in quiescent HeLa cells by exposure to PDBu for 24 hours. This treatment has been shown to abolish the ability of PMA to stimulate phosphorylation of an acidic 80-kDa protein, a presumptive PKC substrate. Consistent with our results with GF 109203X, PDBu completely abolished induction of TF mRNA in response to PMA but had no effect on the induction of TF mRNA by serum (Fig 8). These data indicated that activation of PKC was required for PMA induction of TF mRNA in HeLa cells but did not play a role in serum induction. To determine whether inhibition of PKC also abolished PMA induction of the TF promoter, HeLa cells transfected with pTF(-111)LUC were stimulated with PMA in the presence and absence of GF 109203X. PMA induction of this plasmid was completely abolished by this PKC inhibitor but had no effect on serum induction (data not shown). Therefore, inhibition of PKC prevented induction of the TF promoter by PMA and thus abolished the observed increases in steady-state levels of TF mRNA.

**Discussion**

This study demonstrated that TF activity, mRNA, and gene transcription were all induced in quiescent, transformed epithelial cells in response to serum or PMA. Stimulation of cells with serum and growth factors results in the activation of phospholipase C, which hydrolyzes the substrate, phosphatidylinositol-
4,5-bisphosphate, to generate diacylglycerol and inositol triphosphate. Diacylglycerol stimulates PKC, whereas inositol triphosphate mobilizes intracellular Ca\(^{2+}\) stores. These signaling pathways have been shown to be involved in the induction of many primary response genes by growth factors, serum, and PMA.\(^{32}\) In this study we demonstrated that blocking intracellular Ca\(^{2+}\) mobilization in HeLa cells abolished serum and PMA induction of TF mRNA, whereas downregulation of cytosolic PKC activity or specific inhibition of PKC abolished PMA induction but had no effect on serum induction. Similar results were recently reported by Taubmann and colleagues\(^{40}\) studying serum, growth factor, and PMA induction of TF expression in rat vascular smooth muscle cells. In contrast, downregulation of PKC reduces or abolishes serum and growth factor induction of other primary response genes, such as c-fos and c-myc,\(^{36,38}\) suggesting that serum and growth factor induction of TF gene expression is regulated by alternative signaling pathways.

Phorbol esters induce TF expression in a variety of cell types, including endothelial cells and monocytes.\(^{8,41,42}\) We speculated that this induction in HeLa cells was mediated by the two AP-1 sites in the TF promoter. However, despite AP-1 binding to these sites, functional analysis of the TF promoter in transfected HeLa cells indicated that they were not required for PMA induction of TF gene expression. Further studies are necessary to elucidate the role of these AP-1 sites in the regulation of the TF gene.

Here, we have demonstrated that cis-acting DNA elements required for serum and PMA induction of the TF promoter in HeLa cells were localized to a region between -96 and +121 (relative to the transcription start site). This region included putative binding sites for several transcription factors, including Sp1, EGR-1, AP-2, and late SV40 factor (LSF)\(^{31-34}\) (Fig 4). The role of an overlapping EGR-1/Sp1 site at -70 bp was examined because a similar overlapping EGR-1/Sp1 site was previously shown to function as both a negative and positive regulator of the murine adenosine deaminase promoter.\(^{35}\) However, mutation of the EGR-1 site alone or simultaneously with the Sp1 site did not reduce serum or PMA induction of the TF promoter. In addition, the region between -111 and -39 bp failed to confer serum or PMA inducibility to a heterologous promoter. Recent DNase I footprinting analysis of the TF promoter has revealed a protected 29-bp GC-rich region between -22 and +7\(^{43}\) that includes a putative binding site for a transcription factor termed LSF.\(^{44}\) Serum stimulation of quiescent mouse fibroblasts increases LSF binding to the SV40 promoter,\(^{34}\) suggesting that it may play a role in serum induction. Moreover, a putative AP-2 site at -60 may be involved in serum and PMA induction of TF expression because AP-2 sites mediate induction by phorbol esters.\(^{44}\) Our recent studies indicate that serum and PMA stimulation of quiescent HeLa cells activates a nuclear complex that binds to a region of the TF promoter between -111 and +14 bp (data not shown). The identity of this transcription factor is currently under investigation, and future studies will determine its role in the regulation of TF gene expression.

The TF gene is highly regulated and exhibits cell-specific and inducible expression.\(^{18}\) This present study demonstrated that serum and PMA induction of TF expression in epithelial cells requires a region between -96 and +121 bp that spans the minimal promoter. In contrast, cis-acting regulatory elements that mediate LPS induction in monocytic cells have been localized to an upstream region of the TF promoter, between -227 and -172, which contains two AP-1 sites and a x\(\beta\)-like site.\(^{21}\) Furthermore, Petersen and colleagues\(^ {41}\) concluded that induction of TF expression in endothelial cells by LPS and cytokines required PKC activation. In
fact, our recent studies indicate that LPS- and cytokine-induced TF expression in endothelial cells requires NF-κB/Rel activation. Thus, regulation of TF gene expression in different cell types appears to be mediated by distinct regions of the TF promoter. Induction of TF in response to growth factors, cytokines, oxidized low-density lipoproteins, and tumor promoters suggests that this receptor may play a role in processes other than hemostasis, such as cell proliferation during atherosclerosis and after vascular injury. Elucidation of how TF is induced by serum and growth factors may facilitate rational approaches to intervention in thrombotic and vascular diseases.

Acknowledgments

This work was supported by National Institute of Health grants HL-16411 (Drs Mackman and Edgington) and a research fellowship (Dr Parry) from the American Heart Association, California Affiliate, San Diego County Chapter. This is manuscript 8318-IMM from The Scripps Research Institute. We acknowledge Linda Curtiss for critical review of the manuscript; William Maske, Laura Fuchs, and Pascale Nantermet for technical assistance; and Barbara Parker and Dahlie Dawson for preparing the manuscript.

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Arterioscler Thromb Vasc Biol. 1994;14:807-814
doi: 10.1161/01.ATV.14.5.807

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