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Ets transcription factor binding site is required for positive and TNFα-induced negative promoter regulation

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ABSTRACT

Thrombomodulin (TM) is expressed on vascular endothelial cells and plays an important role in the anticoagulant pathway by maintaining the thrombo-resistance of the blood vessel wall. We show that in primary human endothelial cells TM gene expression is repressed at the transcriptional level by Tumour necrosis factor (TNFα) through a protein kinase C independent pathway. The TM promoter is highly active in endothelial cells and is inhibited by TNFα. The −76/−56 region mediates both specific high basal activity and TNFα-repression. It binds a nuclear factor specific to endothelial cells, that appears to belong to the Ets-family by various criteria. The −76/−56 region contains three direct repeats of the ets-core sequence GGAA that are important for specific high basal activity, TNFα repression and trans-activation by expression of Ets-1 and 2. Although human Ets-1 (h-Ets-1) and chicken c-Ets-1 and 2 stimulate the TM promoter through the −76/−56 element, their activity is not suppressed by TNFα. c-Ets-1 competes and overrides TNFα repression in a concentration dependent manner. We propose that either a different member of the Ets domain protein family, or an Ets-associated co-factor, is the target of the TNFα signalling cascade in endothelial cells.

INTRODUCTION

Tumour necrosis factor (TNFα) is part of a cytokine network implicated in both normal and inflammatory processes (1). Most of its pro-inflammatory effects are due to effects on growth and differentiation of both the immune system and 'reticuloendothelial cells' such as the vascular endothelium that regulates haemostasis (2, 3). TNFα alters the levels of various vascular endothelial surface components, such as leukocyte-adhesive receptors (4) and various blood-coagulation enzyme receptors like tissue factor and thrombomodulin (5, 6). It modulates transcription through different signal transduction pathways to the nucleus. TNFα activates or induces the synthesis of transcription factors, including the proto-oncogenes c-fos, c-jun (7, 8), and NFkB (9, 10, 11, 12), most likely by a protein phosphorylation cascade (13).

We investigated TNFα regulation and cell specific TM gene transcription in human endothelial cells, including primary cultures from umbilical vein (HUVEC) and a continuous cell line EA.hy 926, which is a hybrid between HUVEC and the lung tumour cell line A549 (14, 15). In this paper we show that high basal transcription and TNFα repression of the TM promoter in endothelial cells is directed by a binding site for the Ets domain protein family. However, expression of exogenous chicken c-Ets-1 and human h-Ets-1 overrides TNFα-induced repression in a concentration dependent manner, suggesting that Ets1 competes for the target of the TNFα signalling cascade, which could be either an Ets-1-associated cofactor or a different Ets-like factor.

MATERIALS AND METHODS

Cell culture

Hela and A549 cells were cultured in Dulbeco's modified Eagle's medium (DMEM) plus 10% fetal calf serum. Human umbilical vein endothelial cells (HUVEC) were prepared by the standard method (16) and cultured in Waymouth medium (Gibco), 20% fetal calf serum, 1% crude endothelial growth supplement from bovine retina (17, 18), 100 μg/ml penicillin-streptomycin at 37°C and 5% CO_2, and used non-confluent at passages 2 to 4. The EA.hy 926 (HUVEC−A549 hybrid) cell line was maintained as an adherent monolayer in Iscove's modified Dulbecco's medium (Gibco), 10% fetal calf serum and HAT (12 μg/ml hypoxanthine, 1 μg/ml aminopterin, 8 μg/ml thymidine; Sigma). Endothelial cells were identified by their typical morphology, immunofluorescence staining for von Willebrand factor (17, 19) and expression of thrombomodulin (20).

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Transient transfection and CAT reporter plasmids

Re-plated cells (1×10^6 per 10 cm dishes) were exposed for 16–20 h (Hela, A549) or 6–8 h (HUVEC, EA.hy 926) to the calcium phosphate precipitate (21), refed with fresh medium (+/− TNFα) and harvested after a further 48 h (Hela, A549) or 24 h (HUVEC, EA.hy 926) incubation. All transfections included 10 μg reporters, 5 μg RSV-lacZ (22) as an internal control, and pUC18 to 20–25 μg total DNA. CAT activity was measured according to Gorman *et al.* (21, 22), and expressed relative to CAT-CON.

A 1.4 kb SmaI TM fragment (24) was cloned in pUC 18 (pUC-1.4TM) and shown by sequencing to contain 1100 bp of 5′-flanking region and part of the first exon. This parental plasmid was used to construct reporters in a promoter-less derivative of pCAT-Basic (Promega). Progressive TM 5′-deletion mutants were generated by unidirectional exonuclease III digestion of −1100/+137TM-CAT. Internal deletion dPyPu-192TM-CAT: PCR products (−192/−72/EcoRI and EcoRI/−58/+138 were co-ligated into pCAT-Basic. STR-TM-CAT: PCR products HindIII/−192/−81/ + BglII-TCGAGCAGGAAGCATTTCC-TGGTCGATGATCAGAAGGGCTGATGCCGCAT (26 bp STR-palindromic Ets site) plus −55/+135/XbaI ligated in HindIII-XbaI of pCAT-Basic. Double point mutations (GGAA to CTAA) were generated by PCR amplification with the corresponding mismatch primers and ligated into the HindIII-XhoI sites of −192TM-CAT. TK-CAT constructs: synthetic oligonucleotides ligated in HindIII of tk-CAT (25). PyPu: (sense strand) AGCTTAGGCACTTCCTTCCTTTTCCCGAACGTA.

The recombinant TM-CAT and TM-tk-CAT plasmids were verified by chain termination sequencing (26). All TM specific PCR primers correspond to the published TM genomic sequence (27) and are available on request. CAT-Control (CAT-CON, Promega) has the SV40 promoter/enhancer.

Determination of RNA by Northern analysis and nuclear run-on transcription

RNA from HUVEC (20 μg per lane) was isolated according to Chomzynski and Sacchi (28), transferred to nylon membranes (NEN), immobilised by UV crosslinking (29) and probed as described (30). Protein Kinase C inhibitors (H7, staurosporin) and phophatase inhibitor ocadaic acid were from Gibco-BRL).

Isolation of nuclei and nuclear run on assays were performed according to Clayton and Darnell (31).

Nuclear extracts and DNA binding studies

Nuclear extracts were prepared according to standard methods (32, 33) with minor modifications. Nuclei were incubated with lysis-buffer containing 0.8 M NaCl (HUVEC, EA.hy926 cells)

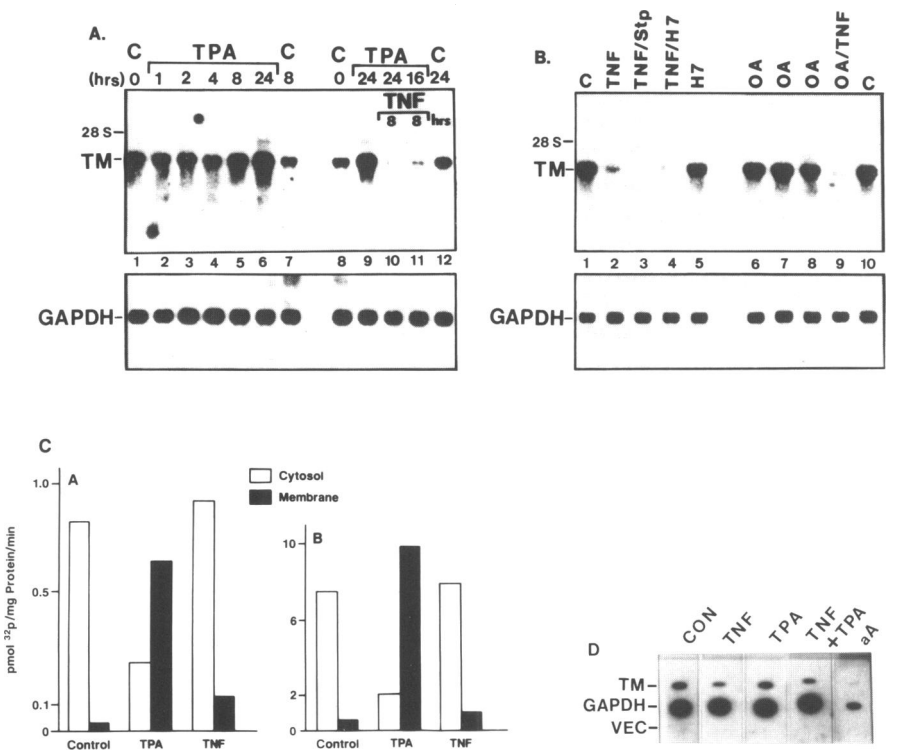


Figure 1. Analysis of human TM RNA levels, PKC activity and TM gene transcription in HUVEC. (A and B) RNA blots probed with TM and GAPDH. **(A)** HUVEC that were either left untreated (C, control), or incubated for the indicated period of time with 100 ng/ml TPA either alone (lanes 2–6 and 9) or costimulated with 100 ng/ml TNFα (lanes 10 and 11). **(B)** HUVEC were incubated for 8 h in medium alone (C, lanes 1 and 10) or with the indicated additions. TNF:TNFα alone. TNF/Stp, TNF/H7: TNFα + the protein kinase inhibitors staurosporin (stp, 150 nM) or H7 (50 μM), respectively. H7: H7 alone (lane 5). OA: 125 μM phophatase inhibitor ocadaic acid for 2, 4 and 8 h (lanes 6–8). OA/TNF: ocadaic acid + TNFα for 8 h (lane 9). With staurosporin, H7 and ocadaic acid there was a preincubation for 30 min prior to TNFα treatment. **(C)** PKC activity in HUVEC and EA.hy 926 cytoplasmic and membrane fractions, following treatment of cell monolayers for 30 min with either medium alone (control), 100 ng/ml TPA or 100 nM TNFα. The data shown are the means of two independent experiments. **(D)** Nuclear run-on transcription assay. HUVEC were exposed for 6 h to either TNFα (TNF), 100 ng/ml TPA (TPA), TPA for 24 h before co-stimulation with TNFα (TNF+TPA), 1mg/ml α-amanitin (aA) or left untreated (CON). ^{32}P-labeled RNA was hybridized to nitrocellulose bound TM or GAPDH cDNAs, or to a control plasmid DNA (Vec), and autoradiographed.

or 0.42 M NaCl (Hela, A549). Yields and protein concentrations: HUVEC, EA.hy 926, 0.1 mg/10^7 cells, 3–6 mg/ml; Hela, A549, 0.5 mg/10^7 cells; 10–15 mg/ml.

Gel retardation, DNase I and methylation protection experiments were essentially performed as described (34, 35). Gel retardation probes (upper strand): TM-3 (labelled probe) 5′-AATTCAGCCCTTCCCACCAGGCACTTCCTTCCTTTTCCC-3′ (TM promoter positions −90/−48). Competitors: PyPu-box (−79/−59) 5′-AATTCCCAGGCACTTCCTTCCTTTTCCCGAAG-3′ (TM promoter positions −79/−59, PyPu-box). Ets site from stromelysin promoter (36): 5′-AATTCGTCAGTTAAGCAGGAAGTGACTAACG-3′. 3xmut: 5′-AGCTTACCAGGCACTTagTTagTTTTagCGAA-3′ (all three TTCC motifs mutated). SP1 (S): 5′-ATTCGATCGGGGCGGGGCGAG-3′. AP2 (A): 5′-GATCGAACTGACCGCCCGCGGCCC-3′.

Primer extension

50 μg total cellular RNA, isolated from EA.hy 926 cells or transiently transfected cells, or tRNA (as a control), were hybridised overnight to body-labelled TM-specific primer (DA-159fi, corresponding to TM non-coding strand +123/+104) or body-labelled CAT-specific primer (DA-182fi, corresponding to CAT non-coding strand 2330/2350 of pCAT-Basic plasmid) at room temperature in 40 mM PIPES pH 6.4 + 1 mM EDTA + 0.4 M NaCl + 80% formamide, ethanol precipitated, extended with 50 U AMV reverse transcriptase (Promega) at 42°C for 1 h and the reaction products were analysed in denaturing 8% acrylamide gels.

Subcellular fractionation and protein kinase C assays

HUVEC (third passage) and EA.hy 926 cells were homogenised, centrifuged, and PKC from the plasma membrane and cytosolic fractions were purified by DEAE-Sephacel chromatography (37). PKC was assayed by two different standard methods, using either the C-terminal N-bromosuccinimide cleavage peptide of histone IIIS (38), or the synthetic peptide AC-MBP(4–14) from myelin basic protein [(39); PKC assay system from Gibco-BRL]. Specificity was confirmed using 20 μM PKC pseudo substrate peptide [PKC19–36 (39)]. Both assays gave comparable results.

RESULTS

TNFα-mediated transcriptional repression does not act through protein kinase C (PKC)

Northern blot analysis revealed a TNFα dependent decrease of TM mRNA (89%, Fig. 1B, lanes 1 and 2). A variety of results showed that TNFα repression is probably not mediated by PKC. Stimulation of PKC with TPA (12-O-tetra-decanoylphorbol 13-acetate, 100 ng/ml) in early passage HUVEC gave only a moderate 2 to 3-fold biphasic increase of TM mRNA (Fig. 1A, lanes 1–7), showing that PKC has little effect on TM mRNA levels. TNFα decreased TM mRNA levels even when PKC was down-regulated (40) to undetectable levels (in either membrane or cytoplasmic fractions, data not shown) with 100 ng/ml TPA for 16 to 24 h (Fig. 1A, lanes 10 and 11). Similarly, the PKC inhibitors staurosporin and H7 (41) did not block repression (Fig. 1B, lanes 3 and 4). PKC was not activated by TNFα in either HUVEC or EA.hy 926 (unlike other cell types, 7), but was activated by TPA, with a significant decrease in the cytoplasmic fraction and an increase in the membrane fraction (Fig. 1C). Inhibition of protein phosphatases 1 and 2A with ocadaic acid (42, 43) did not abolish repression (Fig. 1B, lanes 6–10).

We used an *in-vitro* RNA elongation assay to show that TNFα repressed at the level of transcription. TNFα decreased synthesis of RNA hybridising to TM cDNA by about 73% (3.7-fold repression), independently of a 24 h pre-treatment with TPA (Fig. 1D). α-amanitin greatly decreased in vitro transcription, indicating that transcription was by RNA polymerase II (Fig. 1D). This is in agreement with previous reports, demonstrating that the half life of TM mRNA does not change considerably after TNFα treatment (49).

Promoter sequences required for basal and TNFα regulated TM gene expression

To study regulation of TM gene transcription, we recloned the human TM promoter region (44) and used it to construct reporters containing the chloramphenicol acetyltransferase (CAT) gene. In both early passage HUVEC and EA.hy 926 cells, reporters with propgressive 5′- deletions from −1100 to −74 gave high basal levels of CAT activity (Fig. 2). Deletion to −56 (Fig. 2A) decreased basal activity (85–92%, on average), further deletion to −39 had no supplementary effect, whereas additional removal

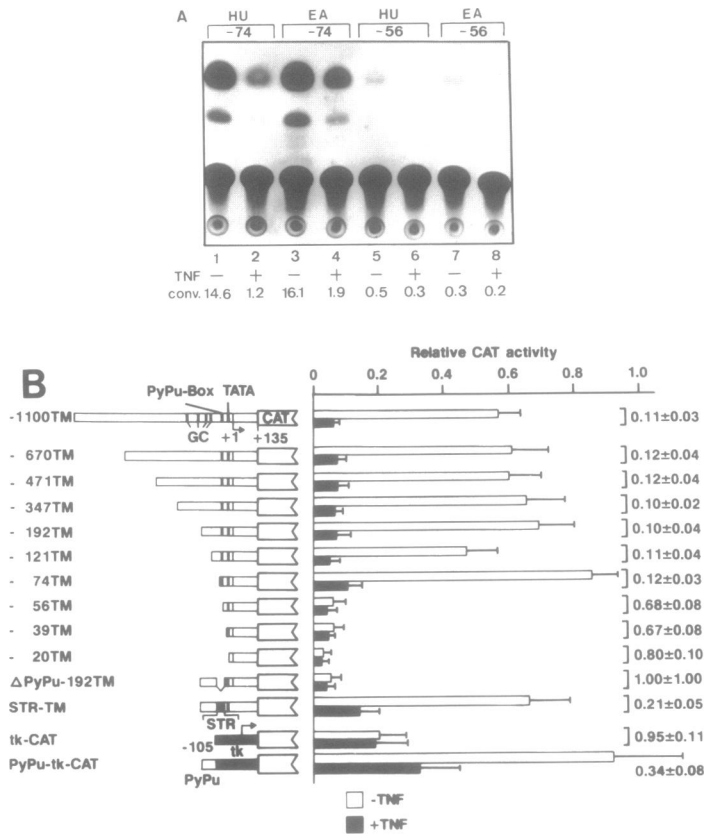


Figure 2. Basal activity and TNFα-induced repression of the thrombomodulin promoter in HUVEC. (A & B) TM-CAT constructs were transiently transfected into HUVEC in the presence (+) or absence (−) of TNFα. **(A).** Autoradiography of CAT assays. **(B)** Summary of basal CAT expression and TNFα repression with the indicated reporter constructs. All CAT activities are beta-galactosidase standardized and expressed relative to CAT-Control. The values on the right are the mean ratios of CAT activity in the presence and absence of TNFα and the standard deviations for at least five separate experiments. A relative activity of 1 denotes (on average) 23.5% acetylation.

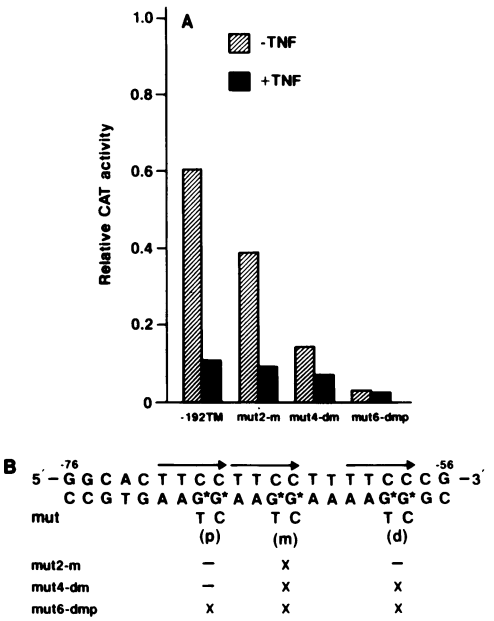


Figure 3. Effect of TM PyPu-box mutants on basal promoter activity and TNFα induced repression. The indicated wild type (−192TM) and double point mutant (mut2-m, mut4-dm, mut6-dmp) reporter plasmids were transfected into EA.hy 926 cells and subsequently treated with TNFα. Averaged values (−/+ 20%) of CAT activities from three different experiments are presented in the histogramme. CAT activities are expressed relative to CAT-CON. A relative CAT activity of 1 denotes (on average) 18% acetylation. (B) Sequence of the −76/−56 PyPu-box of TM promoter. Positions of double point mutations (GG to TC) are indicated by asterisk.

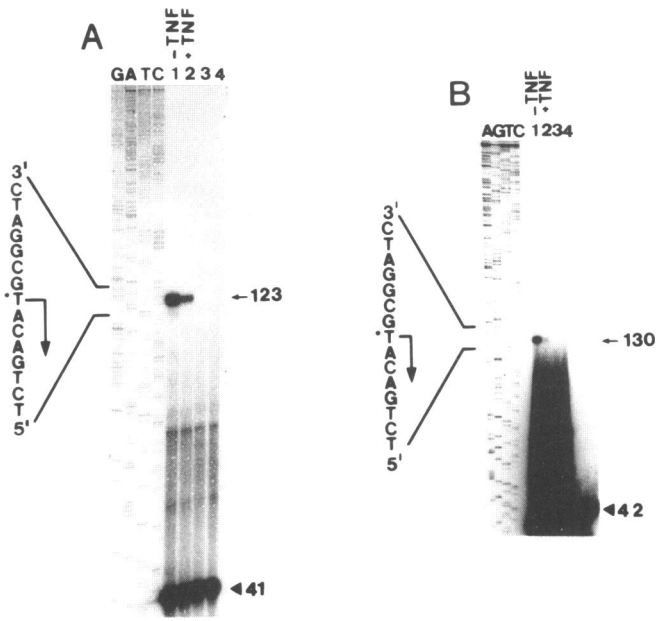


Figure 4. Identification of the in vivo transcription initiation sites of the endothelial TM promoter and the TM-CAT hybrid gene. **(A)** An oligonucleotide (DA-159fi) complementary to the TM sense strand upstream from position +123 was annealed to either total RNA from EA.hy 926 cells that were untreated (−) or treated (+) with TNFα (lanes 1 and 2), RNA from untreated cells preincubated with RNAase A (lane 3), or tRNA (lane 4). **(B)** Reporter plasmid −74/+45TM-CAT was transfected into EA.hy 926 cells. Total RNA was extracted 20 h after transfection +/−TNFα-treatment (lanes 1 and 2, respectively) and the 5'-end and level of CAT mRNA was determined using an oligonucleotide (DA-182fi) complementary to the CAT sense strand. The controls were either RNA from mock-transfected cells (lane 3), or tRNA (lane 4). The sequence surrounding the 5'-ends is shown on the left and the *in vivo* transcription initiation sites (arrows) and sizes of the radiolabeled probes (41nt and 42 nt) are indicated on the right.

of the TATA-Box (−20TM-CAT) lowered the activity even further (98% decrease, Fig. 2B). This indicates that sequences necessary for TM promoter activity are located between −74 and −20, whereas sequences upstream of −74 seem to have a modular effect (Fig. 2B). In HUVEC (Fig. 2B) and EA.hy 926 (data not shown), TNFα strongly inhibited the activity of all constructs lacking sequences upstream of −74 (by about 90%). Progressive deletion from −74 to −56 greatly decreased repression, deletion to −39 had no additional effect, and to −20 abolished inhibition. Most important the −76/−56 sequence, in the absence of other TM sequences, stimulated the heterologous HSV thymidine kinase promoter (25) about 5-fold (Fig. 2B, compare PyPu-tk-CAT and tk-CAT). The antisense orientation gave similar results (not shown), and conferred sensitivity to repression by TNFα (on average 66% decrease, Fig. 2B). In Hela and A549 cells the −76/−56 sequence did not activate the tk promoter, nor confer sensitivity to TNFα (data not shown). In the context of the TM promoter (−192/+135) deletion of −76/−56 (PyPu−192TM, Fig. 2B) decreased promoter activity by about 90%, leaving a residual (but measurable) level that was no-longer repressible by TNFα. These results show that in HUVEC the −76/−56 region is both necessary and sufficient to confer high basal activity and TNFα repressibility.

The −76/−56 region (the PyPu-box) has three GGAA ets-core motifs (Fig. 9). A variety of results show that the Ets motifs mediate the effects of the −76/−56 sequence. A heterologous well characterised ets motif from the stromelysin promoter (36) could functionally substitute for the PyPu-box of the TM promoter. It conferred both high basal activity and significant TNFα suppression when it replaced the −76/−56 sequence of the TM promoter (Fig. 2B, STR-TM). The GG sequence of the Ets core motif (GGAA) is essential for Ets binding. Mutating all three GGs in −192 TM to CT greatly diminished promoter activity (to 10%) and prevented TNFα repression (Fig. 3, mut6-dpm). Mutating the middle GG (mut2-m) reduced CAT activity by about 30%, leaving an activity that was significantly suppressed by TNFα. Mutating the middle and distal GGs (mut-dm) had a greater effect (75% decrease), but left suppressible activity (about 50%). These results suggest that Ets or Ets-like factors are able to mediate both specific positive activation and TNFα repression of the TM promoter in endothelial cells.

Transcripts from TM promoter CAT plasmids are correctly initiated

To identify the endothelial TM gene transcription start site and to show that transcription from the TM-CAT constructs is initiated at the correct sites, primer extension experiments were performed on RNA from untransfected and transiently transfected cells. An oligonucleotide (DA-159fi) that hybridises to TM gene transcripts upstream from position +123 gave the expected 123 nucleotide product [Fig. 4A, (44)]. Furthermore, an oligonucleotide (DA-182fi) complementary to CAT mRNA around the ATG start codon gave the correct 130 nucleotide product. No major primer extension products were generated in mock transfected cells (Fig. 4B, lane 3). TNFα-treatment of EA.hy 926 suppressed correctly initiated transcripts from the TM-CAT plasmids to a similar extent as CAT enzyme activity. These results show that TNFα

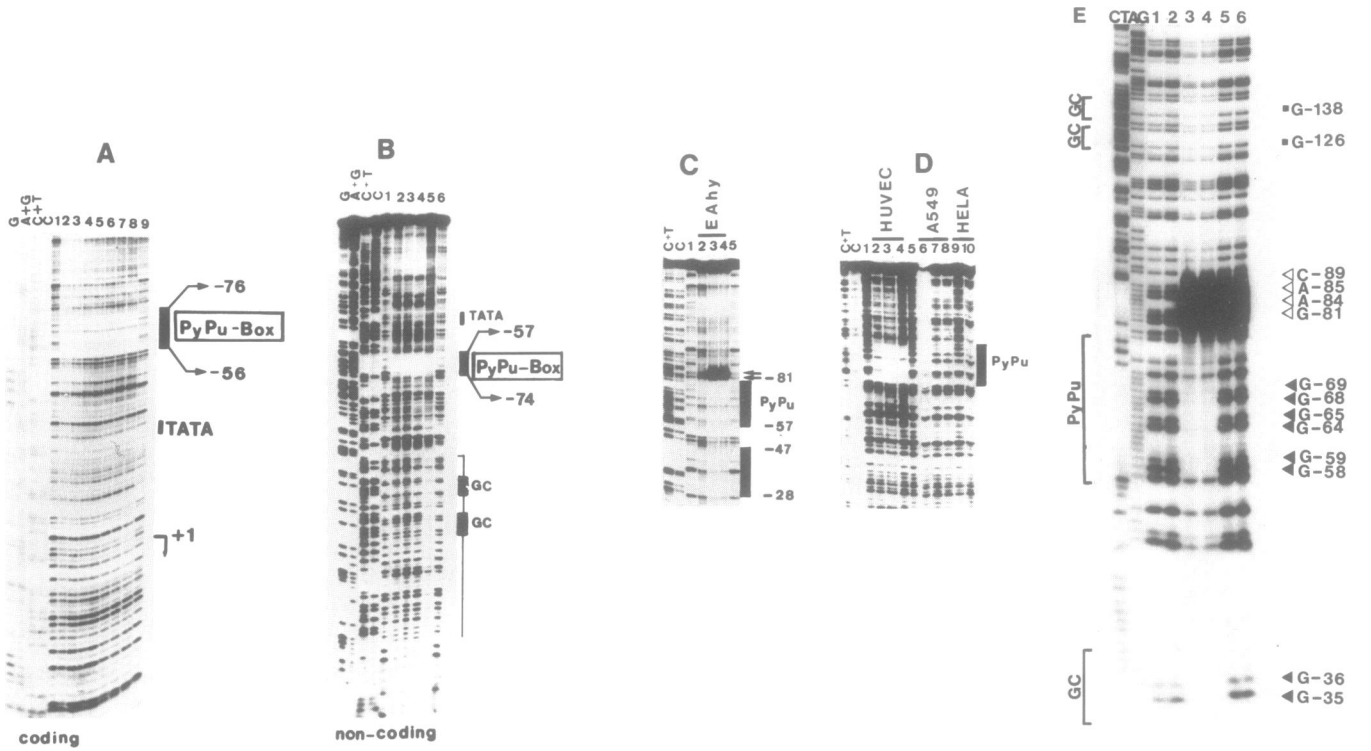


Figure 5. DNase I and methylation protection analysis of TM promoter sequences. **(A)** Footprinting of TM promoter fragment −175/+45 labeled at the +45 (EcoRI) terminus. Lanes 1 and 9: no proteins; lanes 2 to 8: 10.5, 14, 17.5, 21, 24.5, 28 and 35 μg HUVEC nuclear extract. **(B)** TM promoter fragment −192/+137 labeled at the −192 (HindIII) terminus. Lanes 1 and 6: no protein, lanes 2 to 5: 8, 16, 24, and 33 μg HUVEC nuclear extract. **(C)** −175/+45 TM fragment as indicated above. Lanes 1 and 5: no proteins, lanes 2 to 4: 10, 20, and 30 μg nuclear extract from EA.hy 926 cells. **(D)** −112/+45 TM promoter fragment labeled at the +45 terminus. Lanes 1 and 5: no proteins; lanes 2 to 4: 8.8, 17.6, and 36 μg nuclear extract from HUVEC; lanes 6−8: 40 μg A 549 nuclear extract; lanes 9 and 10: 44 and 88 μg Hela nuclear extract. Protected regions are indicated on the right and chemical sequence reactions on the left. **(E)** Methylation protection analysis of the non-coding strand (−18/−192 TM promoter fragment labeled at the −18 (XhoI) terminus. Lanes 1 and 2: methylation in the absence of HUVEC nuclear extract. Lanes 3 to 6: methylation in the presence of 66 μg HUVEC nuclear extract from untreated (lanes 3 and 5) or TNFα treated (lanes 4 and 6) endothelial cells. Lanes 5 and 6: protein−DNA reactions were essentially as in lanes 3 and 4 with binding incubations in the presence of 100-fold excess of cold TM (XhoI-HindIII) competitor. The numbers refer to distance from the transcription initiation site. Filled arrowheads mark positions protected against methylation. Open arrowheads mark hypermethylation sites

suppresses transcription of TM-CAT plasmids from the TM promoter.

Protein−DNA complex formation with the TM promoter

Nuclear proteins from both primary HUVEC and EA.hy 926 cells strongly protected a 21 bp sequence in the −76/−56 region against DNase I digestion (Fig. 4A and B). Weaker footprints were detected around −125 and −140, covering two canonical GC-boxes. EA.hy 926 nuclear extracts additionally protected a region immediately upstream of the TATA-box that resembles a Sp1 binding site (Fig. 5C). We sometimes observed a weak footprint in this region with HUVEC extracts. In contrast to endothelial cells, non-endothelial cell lines (Hela, A549) did not yield a detectable footprint over the most prominent −76/−56 binding site (Fig.5 D).

The −76/−56 protected region contains a 16 bp long purine rich sequence (5′-GGAAAAGGAAGGAAG-3′, lower strand) with three tandem copies of the ets core motif GGAA (45, 46, 47), suggesting that the endothelial specific proteins that bind to this sequence are members of the Ets family. The GG sequence is particularly important for binding by Ets. To assess more precisely the role of each GG sequence for DNA binding we performed methylation protection analysis. All three guanine nucleotide pairs (−58/−59, −64/−65 and −68/−69) were protected against methylation (Fig. 4E), showing that the GGAA core sequence is the essential binding motif within the PyPu-box. There were additional sites of G-protection within the GC-box immediately upstream of the TATA-box (positions −35/−36, Fig. 5E). The 5′-boundary of the PyPu-box was hypersensitive to methylation, as well as DNase I digestion (Fig. 5C, summarised in Fig. 9, and see above). Competition with the homologous unlabeled DNA fragment abolished protection (Fig. 5E, lanes 5 and 6), showing that it was specific. TNFα treated cells gave similar results as untreated cells (Fig. 5E, lanes 3 and 4), showing that inhibition did not result from a decrease in DNA binding activity. They also suggest that TNFα does not induce an alternative factor with different binding characteristics. In gel retardation assays, a specific protein-DNA complex formed with the PyPu box (Fig. 6, lanes 1−4), that was competed by both the unlabeled PyPu-box oligonucleotide and by a well characterised Ets-binding site, but not by a non-specific oligonucleotide [Fig. 6, lanes 6−12, (36)]. Again, TNFα treatment of endothelial cells did not change protein-DNA complex formation over the PyPu-box sequence (data not shown). Furthermore, the TM PyPu-box sequence was specifically bound by nuclear extract proteins from the lymphoid cell line Daudi (48), which is known to contain high levels of Ets proteins (data not shown).

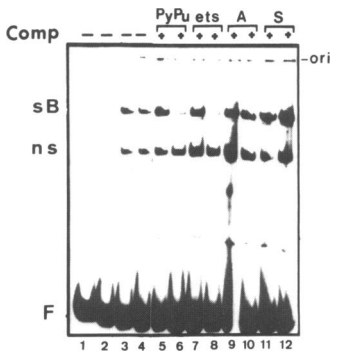


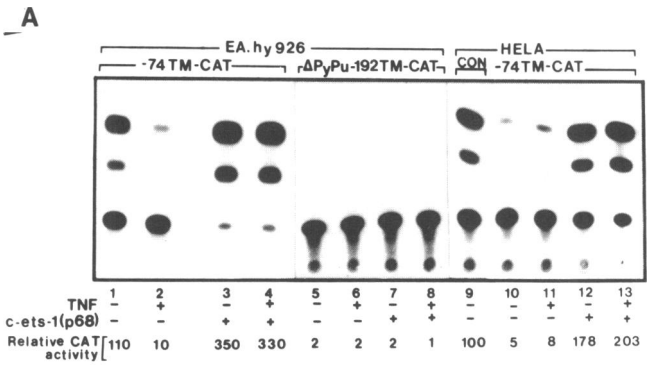
Figure 6. EMSA competition analysis. EMSA performed with TM promoter oligo −90/−48, labeled by Klenow polymerase (10,000 cpm, ~5 fmol). Nuclear extract from EA.hy 926 cells, lanes 1 to 4: 0, 2.5, 5, and 7.5 μg; lanes 5 to 12: 7.5 μg. The specificity of Ets-like binding activity was confirmed by competition with 40 (lanes 5, 7, 9, and 11) or 400-fold (lanes 6, 8, 10, and 12) cold specific (PyPu or ets oligos) or unspecific (A and S oligos) competitors as indicated. sB: specific bound complex. ns: non-specific band. F: free probe.

Ets oncoprotein expression transactivates the TM promoter through the PyPu-box

Cotransfection in EA.hy 926 cells of a c-Ets-1^{p68} expression vector (36) with the −74TM reporter plasmid induced CAT activity 3−4-fold above the high basal level (Fig. 7A, lanes 1, 3). However this induced CAT activity was not significantly decreased by TNFα (lanes 3 and 4). The ΔPyPu−192 TM reporter plasmid was not activated by c-Ets-1^{p68} coexpression (Fig. 7A, lanes 5 to 8), showing that the PyPu-box was required for activation. Essentially identical results were obtained with expression vectors for c-Ets-1^{p54}, c-ets-2 and human Ets-1 (data not shown). In Hela cells expression of exogenous c-Ets-1^{p68} highly induced CAT activity (25-fold, lanes 10, 12), probably because of the low basal activity of the −74TM-CAT reporter plasmid in non-endothelial cells. Again this Ets-dependent CAT induction was not suppressed by TNFα (Fig. 7A, lanes 10 to 13). We obtained similar results with other non-endothelial cells such as A549 and NIH3T3 cells (data not shown). With the PyPu-TM point mutants (see above), mutation of the middle GG had a moderate negative effect on c-Ets-1^{p68} induction (25% decrease compared with the −192 TM wild type, Fig. 7B), whereas mutation of two (mut4-dm) or all three GG copies (mut6-dmp) significantly impaired *trans*-activation (80% and 92% decrease, respectively). Once again, this residual induction of the PyPu-mutant reporter plasmids was not suppressed by TNFα. These results show that Ets expression stimulates the TM promoter through precisely the same sequences that are required for endothelial specific promoter activity, TNFα repression, and binding of endothelial specific factors, suggesting that Ets-like proteins mediate these activities. However, activation by Ets-1 and 2 is not inhibited by TNFα.

Ets overexpression can override TNFα repression in endothelial cells

Cotransfection of STR-TM-CAT and increasing amounts of c-Ets-1^{p68} expression vector in EA.hy 926 cells revealed a competition between ets-dependent promoter activation and TNFα repression (Fig. 8A). Although there was no activation with the lowest amount of c-Ets-1^{p68} expression vector (50 ng), a decrease in TNFα-dependent repression was already detectable (Fig. 8A, compare lanes 1 and 3, lanes 2 and 4). With greater



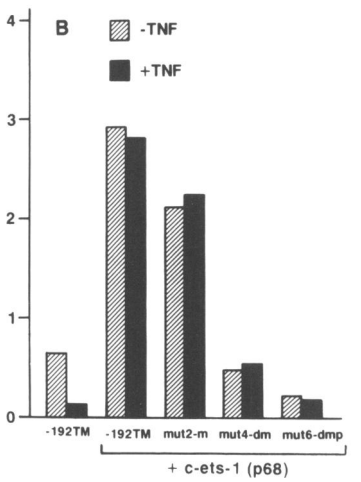


Figure 7. Induction of TM promoter activity by coexpression of c-Ets-1^{p68}. **(A)** The indicated reporter plasmids were cotransfected into EA.hy 926 or Hela cells with 2 μg of c-Ets-1^{p68} (+) or the parental expression vector pSG5 (−) in the absence or presence of TNFα. The CAT activities are given on the bottom. A relative activity of 100 denotes (on average) 24% acetylation. CON: control CAT reporter. **(B)** Influence of GGAA to CTAA PyPu-box mutants on c-Ets-1^{p68} induction of TM promoter. The wild type and mutant reporter plasmids indicated above were cotransfected with the parental expression vector pSG5 or with 2 μg c-Ets-1^{p68} into EA.hy 926 cells in the absence or presence of TNFα. A relative CAT activity of 1 denotes (on average) 17.5% acetylation. Averaged values (± 20%) of CAT activities from three different experiments are presented in the histogrammes. All CAT activities are expressed relative to CAT-Con.

amounts of expression vector there was a progressive increase in activation and loss of sensitivity to inhibition (Fig. 8A and B). With the highest amount of expression vector (2.5 μg) there was a 3−4-fold induction and no suppressive effect by TNFα (Fig. 8A, lanes 13 and 14). The −192TM-CAT reporter gave similar results (Fig. 8B), showing that the PyPu-box of TM and the palindromic Ets binding site of the stromelysin promoter are functionally interchangeable. In Hela cells, the −192TM-CAT reporter gave a value of approximately one for relative CAT activies, irrespective of the c-Ets-1^{p68} expression vector concentration (Fig. 8B). These data suggest that other Ets-like factors, or limiting Ets-1 associated factors, mediate TM promoter repression by TNFα.

DISCUSSION

TNFα down-regulates TM surface expression in endothelial cells in the absence of protein synthesis (49, 50). We have localised a 21 bp sequence of the TM promoter, the PyPu-box, that is

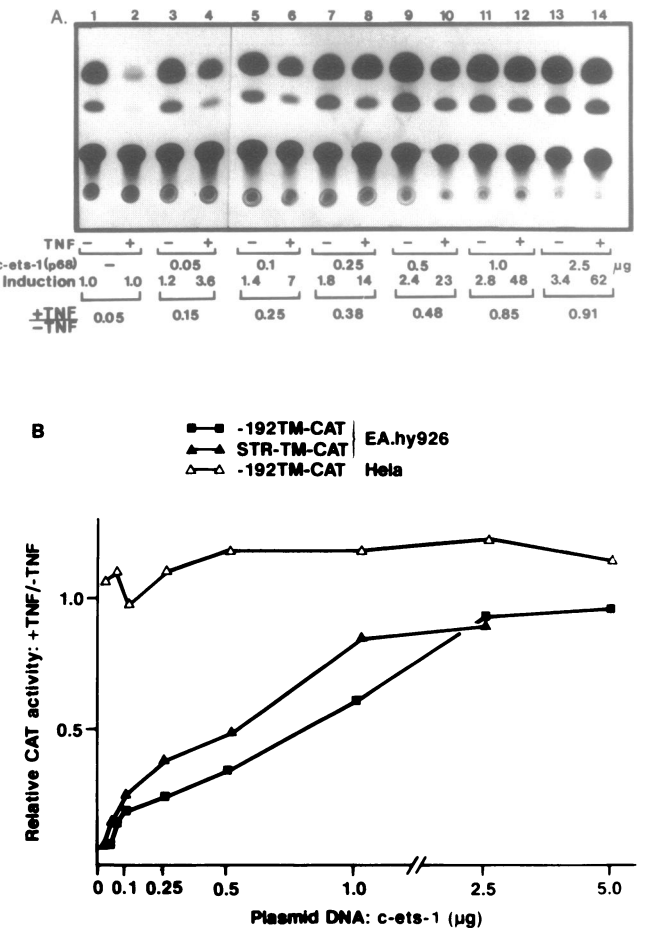


Figure 8. TNFα suppression of the hybrid STR-TM and the native TM promoter is competed by increasing amounts of co-expressed c-Ets-1^{p68} in endothelial cells. **(A)** The reporter construct STR-TM-CAT, which has the TM PyPu-box sequence replaced by the Ets transactivation site of the rat stromelysin promoter (36, Fig. 2B), was either co-transfected with the parental expression vector pSG5 (lanes 1 and 2) or with increasing amounts of c-Ets-1^{p68} in the presence (+) or absence (−) of TNFα. CAT activities from one representative experiment are shown. The relative values for c-Ets-1^{p68} induction and +TNFα/−TNFα ratio given above are based on CAT activities expressed relative to CAT-CON. **(B)** The graph shows the relationship between the amount of cotransfected c-Ets-1^{p68} expression vector and the decreased suppressive effect of TNFα that is observed in EA.hy 926 but not in Hela cells. The −192TM-CAT and STR-TM-CAT reporter constructs were transfected into EA.hy 926 or Hela cells (−192TM-CAT only) with increasing amounts of c-Ets-1^{p68} expression vector in the absence or presence of TNFα. Averaged values of +/− TNFα CAT activities (+/− 15%) from three separate transfections are presented in the graph.

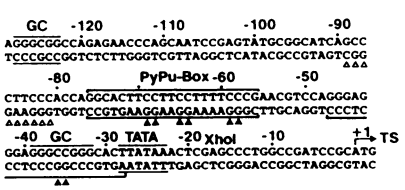


Figure 9. Nucleotide sequence of the human TM promoter. The DNase I footprints generated by nuclear extract proteins from endothelial cells are indicated by brackets, the filled triangles point to the G residues that are protected against methylation, and the open triangles indicate the region of hypermethylation. GC- and TATA-boxes are indicated.

essential for both high basal activity in endothelial cells and TNFα negative regulation. Three GGAA core binding motifs of the TM PyPu-box are required for maximal promoter activation, although two are sufficient for 80% of the activity. This is in agreement with previous reports showing that more than one GGAA core sequence is required for transactivation by c-Ets-1 and 2 (36). DNA binding and transfection studies suggest that a constitutively active Ets-like transcription factor is the downstream target for TNFα in endothelial cells. Recently it has been shown that c-Ets-1 is predominantly expressed in proliferating endothelial cells *in vivo* and *in vitro* (51, 52, J.Otte, U.Knies, and D.v.d.Ahe, manuscript in preparation). Although c-Ets-1 expression stimulates the TM promoter through the TNFα sensitive element, TNFα does not inhibit its activity. Rather, c-Ets-1 over-expression can override repression. Several models can account for our data.

The first possibility is that the endothelial Ets-like transcription factor is c-Ets-1, but the target for TNFα is an Ets-1 associated factor. This would explain the following data: (i) Both the TM PyPu-box and an established good c-Ets-1 binding site are down-regulated by TNFα. (ii) Over-expression of c-Ets-1 prevents TNFα-repression, presumably by competing out a limiting Ets associated factor. (iii) There is a low basal activity and lack of inhibition by TNFα in non-endothelial cells, which can be explained by the lack of c-Ets-1, and supposedly of the putative co-factor, in these cells. There is a growing body of evidence that Ets domain transcription factors can interact with other DNA binding or non-DNA binding gene regulatory factors (53). Association of AP1 and Ets DNA binding sites has been found in several viral and cellular genes (54, 55, 36). The Ets family members Elk-1 (56) and SAP-1 (57) form complexes with SRF (58). Both Ets-1 and Ets-2 may interact with a common co-activator through their transactivation domains (59).

There are other explanations for our results. c-Ets-1 expression may induce or suppress transcription of a second gene, which interferes with TNFα-induced gene suppression in endothelial cells. Alternatively, c-Ets-1^{p68} may directly modulate the TNFα-induced signalling cascade further upstream, for instance by interacting with certain nuclear or cellular components of the pathway. Finally, and perhaps most simply, the Ets-like factor expressed in endothelial cells and involved in TM gene regulation may be a different member of the family. The target for TNFα could be a sequence that is less conserved than the DNA-binding (Ets) domain.

Ets proteins are able to change gene expression in response to extra-cellular signals. Phosphorylation inhibits non-specific DNA binding by Ets-1 (60) and stimulation of PKC increases both the stability (61) and expression (62) of Ets-2. PKC is probably the mediator of TNFα activation of two widely distributed transcription factors, NFkB (11) and AP1 (7), which are expressed in endothelial cells (63). However, our results show that PKC most likely does not mediate TNFα down regulation of the TM promoter in endothelial cells, even though PKC is present. Potential mediator kinases are Raf-1 and MAPK, that activate AP1/Ets driven promoters (64, 65). We did not observe any influence of TNFα on binding of the endothelial nuclear factor(s), suggesting that the mechanism does not involve loss of DNA binding by mechanisms such as phosphorylation.

The identification of an endothelial Ets-target gene reported here provides an opportunity to study the role of Ets proteins in endothelium and blood vessel function. Endothelial cells play a major role in modulating normal vascular function and

pathology associated with angiogenesis, developmental processes, wound healing, thrombosis, and vascularization of tumours. They are an important target for TNFα mediation of shock and diffuse tissue injury. Cytokine dependent transcriptional suppression and activation of key proteins of the vascular endothelium is of fundamental importance in regulation of hemostasis. Our results point to a key role for Ets transcription factor proteins as important mediators of inflammatory signalling cascades in vascular cells.

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