Transcriptional Regulation of the Tissue Factor Gene in Human Epithelial Cells Is Mediated by Sp1 and EGR-1*

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Mei-Zhen Cui‡§, Graham C. N. Parry‡, Paul Oeth, Heike Larson, Melissa Smith, Ruo-Pan Huang, Eileen D. Adamson, and Nigel Mackman†

From the Department of Immunology, The Scripps Research Institute and La Jolla Cancer Research Foundation, La Jolla, California, 92037

Tissue factor (TF) gene expression is rapidly induced in epithelial cells by phorbol 12-myristate 13-acetate and serum. We have shown that this induction is mediated by a novel serum response region (SRR) (−111 to +14 bp) within the human TF promoter. In this study, we characterized cis-acting genetic elements within the SRR that regulated basal and inducible expression of the TF gene in HeLa cells. Gel mobility shift assays using oligonucleotides spanning the entire SRR identified three 12-base-pair (bp) motifs within subregions 1, 2, and 3 that bound constitutively expressed Sp1 and inducibly expressed EGR-1. Analysis of protein binding to these 12-bp motifs by competition with Sp1 and EGR-1 sites, mutation, and antibody supershift experiments indicated that they each contained distinct EGR-1 and Sp1 sites that overlapped by 6 bp. Functional studies using HeLa cells transfected with plasmids containing the wild-type TF promoter (−111 to +14 bp) or derivatives containing mutations in the three Sp1 and/or EGR-1 sites examined basal and inducible expression. The Sp1 sites mediated basal promoter activity, and both Sp1 and EGR-1 sites were required for maximal induction of the TF promoter by phorbol 12-myristate 13-acetate or serum. These data indicated that TF gene expression in HeLa cells was regulated by both Sp1 and EGR-1.

Tissue factor (TF) is the primary cellular initiator of the coagulation protease cascades (1). The TF gene is expressed in a cell type-specific manner in vivo (2). For example, TF mRNA is expressed in the upper stratum granulosum layer of the epidermis but not in the dermis (3). In vitro studies defined the TF gene as an immediate early gene because it is induced in quiescent fibroblasts and epithelial cells by serum and purified growth factors in the absence of de novo protein synthesis (4–9). However, the time course of induction of TF mRNA, as well as c-myc mRNA, is delayed compared with the induction of other immediate early genes, including c-fos and egr-1 (4). Our previous studies show that serum and phorbol 12-myristate 13-acetate (PMA) induction of the human TF gene is mediated by a region of the TF promoter (−111 to +14 bp relative to the start site of transcription) called the serum response region (SRR) (9, 10). This SRR does not contain DNA sequences resembling the serum response element characterized in the promoter of the c-fos gene and other immediate early genes (11, 12). Deletional analysis and mutagenesis of the SRR failed to identify a single cis-acting regulatory element that mediated induction (9) in epithelial cells, suggesting that several distinct regions may act in concert to regulate inducible TF gene expression.

The SRR of the TF promoter includes a 12-bp motif that contains putative overlapping EGR-1/Sp1 binding sites (9). The EGR-1 and Sp1 sites contain six overlapping nucleotides, suggesting that binding of each transcription factor is mutually exclusive. Sp1 and EGR-1 both contain three zinc finger motifs of the Cys-2-His subclass that bind to nucleotide triplets within their respective sites (13, 14). Importantly, EGR-1 does not compete Sp1 binding to a consensus Sp1 site (15, 16). In addition, methylation interference studies indicate that the two proteins exhibit different contact sites within a 12-bp DNA sequence containing overlapping EGR-1/Sp1 sites (17). Similar 12-bp motifs have been reported in the egr-1 gene itself (18), the murine adenosine deaminase gene (19), the human synapsin I gene (20), and the homeobox containing gene hox-1.4 (21).

Sp1 is a general transcription factor that activates transcription of a subset of genes containing Sp1 sites (22, 23). The Sp1 gene is constitutively expressed in HeLa cells (24–26). In contrast, the egr-1 gene is rapidly and transiently induced in HeLa cells by serum and PMA (18, 27). EGR-1 (28) (also known as Zif268, NGF1-A, krox24, and TIS8 (29–32)) is a nuclear phosphoprotein that binds to a specific DNA sequence, 5′-GGCGGGCGG-3′, in a zinc-dependent manner (16, 17, 33). EGR-1 has been shown both to activate and repress transcription in transient transfection assays (15, 17–19).

In this study, we determined that Sp1 and EGR-1 bound to three 12-bp motifs within the SRR of the TF promoter, each of which contained overlapping EGR-1/Sp1 sites. Sp1 was constitutively expressed, whereas EGR-1 was induced in response to PMA or serum. Functional studies using the cloned wild-type TF promoter and derivatives containing mutations in the Sp1 and/or EGR-1 sites indicated that Sp1 was required for basal TF expression and that both Sp1 and EGR-1 mediated inducible TF expression.

MATERIALS AND METHODS

Cell Culture and Transfections—HeLa cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA). Cells were serum-starved

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‡ These authors contributed equally to this study.

§ Present address: Dept. of Cell Biology, The Cleveland Clinic, Cleveland, OH 44195.

¶ To whom correspondence should be addressed: Scripps Research Inst., 10666 Torrey Pines Rd., IIMM-17, La Jolla, CA 92037. Tel.: 619-554-8594; Fax: 619-554-6146.

† The abbreviations used are: TF, tissue factor; PMA, phorbol 12-myristate 13-acetate; SRR, serum response region; bp, base pair.
by culturing in Dulbecco's modified Eagle's medium containing 0.5% serum for 48 h before induction with 20% serum or PMA (50 ng/ml (Sigma)). HeLa cells were transfected using DOTAP (Boehringer Mannheim). Supercoiled plasmid DNA was used to transfect each subconfluent cell culture. Transfected cells were serum-starved for 48 h before a 5-h induction with either PMA (50 ng/ml) or 20% serum supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10% insulin-like growth factor (10 ng/ml), and basic fibroblast growth factor (10 ng/ml) (Collaborative Biomedical products, Bedford, MA). Transactivation by EGR-1 was performed by cotransfecting plasmids containing the TF promoter with the plasmid, pSVKrox-24 (kindly provided by P. Charnay), which expresses EGR-1 (17). Luciferase activity was determined using the Luciferase Assay System (Promega Corp., Madison, WI) and a luminometer (Analytical Luminescence Laboratory, San Diego, CA). PCMVp2 (2 μg) (Clontech Laboratories, Inc., Palo Alto, CA) was used as an internal control to assess transfection efficiencies. β-galactosidase activity was measured using the Galacto-Light assay system (Tropix, Bedford, MA). Statistical analysis of the data used Student's t test.

Plasmids—Functional analysis of the TF promoter was performed using plasmids containing the wild-type TF promoter and mutated derivatives cloned upstream of the luciferase reporter gene. First, a Smal–SacI fragment (−111 to +14 bp) of the TF promoter was cloned into pUC19 to create pGTF2368. Second, mutations in the Sp1 and/or EGR-1 sites in the TF promoter were generated by ligating mutated oligonucleotides spanning a subregion between −111 to −56 bp (−55 to −20 bp) spanned by a linker oligonucleotide. 5′-GACGGCGGAGGAACCGGAGGCGGGCTTCGAGCACAGGGGGTTGTCCCATTATAG-3′, and pGTF2368 digested with Smal and BssHII, which contains a subregion between −19 and +14 bp. The wild-type TF promoter (−111 to +14 bp) was subcloned from pGTF2368 into the promoterless expression vector pGL2-Basic (Promega Corp.) to create pTF (−111 to −14 bp)LUC. The same region from three plasmids containing mutations in the Sp1 and/or EGR-1 sites was subcloned into pGL2-Basic to create: (a) pTF(Sp1m)LUC containing the oligonucleotide −111 to −56 bp, 5′-GACGCGGAGGAACCGGAGGCGGGCTTCGAGCACAGGGGGTTGTCCCATTATAG-3′, (b) pTF(EGR-1m)LUC containing the oligonucleotide, −111 to −56 bp, 5′-GGGCCGAGGACGCTTCGAGACACAGGGGGTTGTCCCATTATAG-3′, and (c) pTF(EGR-1m/Sp1m)LUC containing the oligonucleotide, −111 to −56 bp, 5′-GGGCCGAGGACGCTTCGAGACACAGGGGGTTGTCCCATTATAG-3′. Mutated residues are indicated in bold. All plasmids were sequenced to confirm base pair substitutions.

Gel Shift Assays—Nuclear extracts were prepared from 5 × 10⁶ HeLa cells as described (34). Protein concentrations in nuclear extracts were 1–5 mg/ml, as determined by BCA protein assay (Pierce). Oligonucleotides containing a prototypic Sp1 site (underlined), 5′-AATTCGAGGGAGGAGCGGCGGGGGCGATTTCGAGTCA-3′, and an AP-1 site (underlined), 5′-AATTCGAGGGAGGAGCGGCGGGGGCGATTTCGAGTCA-3′, were used as probes. Protein–DNA complexes were identified by incubating nuclear extracts from quiescent and PMA-stimulated HeLa cells with each of the seven radiolabeled oligonucleotides (Fig. 2 and data not shown). Regions 1 and 3 exhibited a similar pattern of protein binding and formed major (I) and minor (II) complexes with unstimulated cells and formed three complexes (I, II, and III) with PMA-stimulated cells. Region 2 formed a single minor complex (I) with unstimulated cells and two complexes (I and II) with PMA-stimulated cells. Similar complexes were obtained using nuclear extracts from serum-stimulated cells (see Fig. 5A). No complexes were observed with region 4 (Fig. 2) or regions 5–7 (data not shown).

Complex I formed using either the region 1, 2, or 3 probes exhibited the same mobility (Figs. 2 and 3D), suggesting that it may represent binding of the same constitutively expressed protein. Similarly, complex II formed with the different probes migrated with the same mobility (Figs. 2 and 3D), consistent with binding of the same inducible protein. Region 2 contains the previously identified 12-bp motif that includes overlapping EGR-1/Sp1 sites (Fig. 1). Inspection of the DNA sequence of regions 1 and 3 indicated that they each contained a similar 12-bp motif (Fig. 1). The EGR-1 site in region 2 exactly matched the proposed EGR-1 consensus binding site and bound large amounts of the inducible complex II compared with regions 1 and 3. The oligonucleotides R2−1, and R2−2 (Fig. 1) exhibited the same pattern of protein binding (data not shown) as the region 2 (R2) oligonucleotide (Fig. 2), indicating that complex II formation did not involve DNA sequences overlapping with regions 1 and 3. Regions 1 and 3 included putative EGR-1 sites with a C at position 1 instead of a G present in the EGR-1 consensus binding site (16, 17, 33) and bound lower levels of the inducible complex II compared with region 2.

EGR-1 and Sp1 Bind to Three 12-bp Motifs in the TF Promoter That Contain Overlapping Sequences—To determine the identity of proteins that bound to regions 2 and 3, we compared the mobilities of these three complexes (I, II and III) with complexes formed using radiolabeled oligonucleotides containing either a prototypic Sp1 site or a prototypic EGR-1 site (Fig. 3A). A single inducible complex (EGR-1) formed using the prototypic EGR-1 site comigrated with complex II formed using region 2 (Fig. 3A, lanes 1–4), and complex II formed using regions 1 and 3 (data not shown). A major (Sp1) and minor complex were formed between the prototypic Sp1 site and nuclear extracts from both unstimulated and PMA-stimulated cells, which comigrated with complexes I and III, respectively, formed using region 3 (Fig. 3A, lanes 5–8) and regions 1 and 2 (data not shown). Complex III and the minor complex observed with the prototypic Sp1 site may represent binding of a proteolytic subfragment of Sp1 that displays the same affinity and
specificity for binding to Sp1 sites (24). These data suggested that the constitutively expressed complexes I and III may represent Sp1 binding to the Sp1 sites in each of the regions 1, 2 and 3 of the TF promoter, whereas the inducibly expressed complex II may represent binding of EGR-1, which is induced in stimulated HeLa cells (18, 27).

Competition studies were performed using various unlabeled oligonucleotides to examine the binding specificity of complex I (designated Sp1), complex II (designated EGR-1), and complex III formed using regions 2 and 3 (Fig. 3B). The Sp1 complex (complex I) and complex III were competed with a Sp1 site but not with an EGR-1 site or AP-1 site. Conversely, the EGR-1 complex (complex II) was competed with an EGR-1 site but not with Sp1 or AP-1 sites. These competition studies further suggested that Sp1 and EGR-1 bound to overlapping EGR-1/Sp1 sites in these regions of the TF promoter.

Next, we analyzed protein binding to oligonucleotides containing base pair substitutions in the EGR-1 sites and/or the Sp1 sites (Fig. 3C). For region 2, these mutated oligonucleotides (Fig. 1) were used to compete the Sp1 and EGR-1 complexes formed between the radiolabeled wild-type oligonucleotide, R2, spanning region 2 and nuclear extracts from PMA-stimulated cells (Fig. 3C, lanes 1–6). Unlabeled wild-type oligonucleotide competed formation of both the Sp1 and EGR-1 complexes. Oligonucleotides containing mutated EGR-1 sites, R2–2 EGR-1m1 and R2–2 EGR-1m2, competed only the Sp1 complex; an oligonucleotide containing a mutated Sp1 site, R2–2 Sp1m, competed only the EGR-1 complex; and an oligonucleotide containing mutations in both sites, R2–2 EGR-1m/Sp1m, did not compete either complex. For region 3, the wild-type oligonucleotide and mutated derivatives (Fig. 1) were radiolabeled and incubated with nuclear extracts from PMA-stimulated cells (Fig. 3C, lanes 7–10). Complexes I (Sp1), II (EGR-1), and III were all observed using wild-type region 3. In contrast, an oligonucleotide containing a mutated Sp1 site, R3 Sp1m, formed only the EGR-1 complex, and an oligonucleotide containing mutations in both sites, R3 EGR-1m/Sp1m, did not compete either complex. For region 1 (data not shown).

Finally, the identity of the proteins present in these complexes was determined using antibodies that specifically recognize the transcription factors EGR-1, Sp1, or AP-2 (Fig. 3D). Indeed, other members of the EGR family can bind to an EGR-1 site (33, 39), but these transcription factors are not recognized by the EGR-1 antiserum used. The EGR-1 complex (complex II) observed using PMA-stimulated cells, and regions 2 and 3 were abolished by preincubation with an EGR-1-specific antibody but not by Sp1- or AP-2-specific antibodies (Fig. 3D). Similar results were observed using region 1 (data not shown). Conversely, the Sp1 complex (complex I) formed using region 3 was abolished by preincubation with an Sp1-specific antibody but not by EGR-1- or AP-2-specific antibodies (Fig. 3D, lanes 5–8). Therefore, we concluded that complexes I and II formed using oligonucleotides spanning regions 1, 2, and 3 represented binding of Sp1 and EGR-1, respectively. Sp1 was constitutively expressed in HeLa cells, and binding was not increased upon
stimulation of the cells. In contrast, EGR-1 binding activity was not detected in nuclear extracts from quiescent HeLa cells but was rapidly induced by stimulation with serum and PMA.

Binding of Recombinant Sp1 and EGR-1 to the 12-bp Motifs in Regions 1 and 2—The EGR-1 and Sp1 sites within the 12-bp motifs contained six overlapping nucleotides, suggesting that EGR-1 and Sp1 binding was mutually exclusive. Gel shift mobility assays performed using nuclear extracts did not show a reduction in Sp1 binding in the presence of inducible EGR-1 because the radiolabeled oligonucleotide probe was in excess. Therefore, to determine if EGR-1 could compete prebound Sp1 under more controlled conditions, we examined binding of recombinant Sp1 and EGR-1 to regions 1 and 2. Analysis of the amount of Sp1 complex formed at 5, 10, and 20 min established that Sp1 binding to these probes was at equilibrium at 20 min. Large amounts of Sp1 bound to region 1 (Fig. 4A, lane 1) compared with region 2 (Fig. 4B, lane 1), consistent with the relative intensities of complex I formed using nuclear extracts from HeLa cells. The wild-type oligonucleotides spanning region 3 (WT; R3) and derivatives containing a mutated Sp1 site, Sp1m (R3 Sp1m), a mutated EGR-1 site, EGR-1m (R3 EGR-1m), or mutated EGR-1 and Sp1 sites, EGR-1m/Sp1m (R3 EGR-1m/Sp1m), were radiolabeled and incubated with a nuclear extract from PMA-stimulated (1 h) HeLa cells. The Sp1 complex and EGR-1 complex II complexes are indicated. D, identification of proteins present in the protein-DNA complexes using specific antibodies. Oligonucleotides spanning regions 2 (R2) and 3 (R3) were incubated with nuclear extracts from PMA-stimulated (1 h) HeLa cells in the absence or the presence of 5 μl of EGR-1, Sp1, or AP-2 antibody (Ab) (Santa Cruz Biotechnology). The Sp1 complex and EGR-1 complex II complexes are indicated. Complexes were separated using 6% nondenaturing acrylamide gels.
similar to those of complex I and II, respectively, formed using nuclear extracts (data not shown). We found no evidence of a slower migrating complex representing a Sp1-EGR-1-oligonucleotide complex. In addition, Sp1 antibodies recognized complex I (Sp1) but did not recognize complex II (EGR-1) formed with either R1 or R2, indicating that Sp1 was not present in complex II (data not shown). However, our data cannot formally exclude the possibility that EGR-1 binds to the pre-formed Sp1 complex via protein-protein interactions to form an EGR-1-Sp1-oligonucleotide complex that dissociates upon electrophoresis. PMA and serum stimulation of HeLa cells induces expression of EGR-1 protein, suggesting that EGR-1 may compete for Sp1 binding to the 12-bp motifs within the TF promoter.

Induction of EGR-1 Expression in HeLa Cells—The kinetics of induction of EGR-1 binding activity was determined using nuclear extracts from HeLa cells stimulated for various times with PMA or serum. Gel mobility shift assays using oligonucleotides spanning regions 2 (Fig. 5A, upper panel) and region 3 (Fig. 5B, lower panel) revealed that EGR-1 binding activity was transiently expressed in PMA-stimulated cells (Fig. 5A). EGR-1 binding activity was not detected in quiescent cells but was first observed 20 min after stimulation and increased to maximal levels between 1 and 2 h before decreasing to undetectable levels at 20 h (Fig. 5A). Similarly, serum stimulation of cells induced EGR-1 expression within 1 h. In contrast, Sp1 binding activity detected using regions 2 and 3 was present in quiescent cells and was not increased by serum or PMA stimulation (Fig. 5A).

To investigate if stimulation of the cells induced de novo synthesis of EGR-1 or activated binding of pre-existing EGR-1 protein by post-translational modification, levels of EGR-1 protein in nuclear extracts from quiescent and PMA-stimulated cells were assessed by Western blotting using an EGR-1 polyclonal antibody (Fig. 5B). No EGR-1 protein could be detected in nuclear extracts from quiescent cells. However, EGR-1 protein expression was induced following stimulation with PMA for 1 h (Fig. 5B). In contrast to the induction of EGR-1 expression, Sp1 protein was constitutively expressed by quiescent HeLa cells (Fig. 5B). The Sp1-specific antiserum recognized two polypeptides of ~105 and 95 kDa in size, which appear to arise from differential phosphorylation of Sp1 (40). The slower migrating species very likely represents the highly phosphorylated form of Sp1 (40). PMA stimulation of the cells did not increase Sp1 levels and did not change the phosphorylation of Sp1 (Fig. 5B). Similar results were seen with serum-stimulated
To exclude the possibility that stimulation of HeLa cells induced pre-existing EGR-1 protein that was present in an inactive cytoplasmic form, we analyzed levels of EGR-1 protein in cytosolic and nuclear extracts from quiescent PMA- and serum-stimulated cells. No EGR-1 protein was detected in cytosolic extracts, but EGR-1 was detected in nuclear extracts from PMA- and serum-stimulated cells (data not shown), indicating that stimulation of cells induced de novo synthesis of EGR-1. However, we cannot exclude the possibility that quiescent HeLa cells contained low levels of pre-existing EGR-1 that were not statistically significant, whereas the reduction observed with pTF(EGR-1m/Sp1m)Luc was statistically significant (p < 0.05). E. TF promoter plasmids (8 μg) were cotransfected into HeLa cells with pSVKrox-24 (8 μg), which expresses EGR-1 (17), or with pUC19 (8 μg) as a control. Cells were serum-starved for 53 h before assaying luciferase activity in the presence and the absence of EGR-1 protein to calculate the fold induction. In each case (C and D) data are shown minus the background of the vector control (pGLO2-Basic, Promega Corp.). In each experiment, values were determined from triplicate wells of a 6-well plate and corrected for transfection efficiency (see "Materials and Methods"). Results from at least four independent experiments are shown (means ± S.E.).
tained mutations in all three Sp1 and all three EGR-1 sites (Fig. 6A). Base pair substitutions chosen to mutate the Sp1 and/or EGR-1 sites abolished protein binding (see Fig. 3C). Luciferase activity directed by the wild-type TF promoter transfected into quiescent HeLa cells was designated as 100%. Mutation of the three EGR-1 sites in pTF(EGR-1m)Luc did not affect basal promoter activity (Fig. 6B). In contrast, mutation of the three Sp1 sites in pTF(Sp1m)Luc and pTF(EGR-1m/Sp1m)Luc reduced basal promoter activity by greater than 90% (Fig. 6B), indicating that Sp1 regulated basal activity of the TF promoter.

Sp1 and EGR-1 Are Required for Maximal Induction of the TF Promoter in HeLa Cells—To determine the role of Sp1 and EGR-1 in inducible expression of the TF gene, we determined the level of PMA or serum induction of luciferase activity mediated by the wild-type TF promoter and mutated derivatives. EGR-1 has been shown to function as both a transcriptional activator and repressor of different genes in various cell types (15, 17–19). PMA stimulation of transfected cells induced luciferase activity expressed by the wild-type TF promoter (Fig. 6C). Mutation of the three Sp1 sites increased the level of PMA induction, whereas mutation of the three EGR-1 sites reduced PMA induction compared with the wild-type TF promoter (Fig. 6C). Simultaneous mutation of all the Sp1 and EGR-1 sites abolished PMA induction of luciferase activity mediated by the TF promoter. Luciferase activity expressed by the wild-type TF promoter was induced 2–2.5-fold by serum. To increase the magnitude of induction for analysis of the mutated derivatives, serum was supplemented with epidermal growth factor, insulin-like growth factor-1, and basic fibroblast growth factor (Fig. 6D). Mutation of either the Sp1 sites or the EGR-1 sites each slightly reduced the level of serum induction compared with the wild-type TF promoter, although these changes were not statistically significant. However, simultaneous mutation of all the Sp1 and EGR-1 sites abolished serum induction of luciferase activity mediated by the TF promoter, and this reduction was statistically significant (p < 0.05) (Fig. 6D). These results suggested that both EGR-1 and Sp1 were required for maximal induction of the TF promoter in response to serum or PMA.

Further studies examined if expression of EGR-1 alone could transactivate the TF promoter in the presence of endogenous Sp1 (Fig. 6E). Plasmids containing the wild-type TF promoter and mutated derivatives were cotransfected with a plasmid, pSVKrox-24, that expresses EGR-1 (17). The wild-type promoter in pTF(−111 to +14)Luc was transactivated by EGR-1. The level of transactivation was increased by mutation of the Sp1 sites in a similar manner to the results observed using PMA. However, mutation of the EGR-1 sites in pTF(EGR-1m)Luc and in pTF(EGR-1m/Sp1m)Luc abolished transactivation mediated by EGR-1. Therefore, EGR-1 acted as a transcriptional activator of the TF gene in HeLa cells.

DISCUSSION

This study demonstrated that three 12-bp motifs within the SRR, each of which contained overlapping EGR-1/Sp1 sites, mediated both PMA and serum induction of the TF gene. Sp1 was constitutively expressed in unstimulated cells, whereas de novo synthesis of EGR-1 was rapidly induced by PMA and serum stimulation. Functional analysis of plasmids containing mutated 12-bp motifs suggested the presence of two transcriptional components requiring either intact Sp1 sites or intact EGR-1 sites, which both contributed to maximal induction of the TF promoter in HeLa cells.

Previous studies defined the TF gene as an immediate early gene (4–7, 9). The data presented here suggested that transcriptional activation of the TF gene in HeLa cells by PMA or serum was in fact composed of both an immediate early component and a delayed early component. We propose that the immediate early component of TF gene induction requires Sp1 sites. Specific mutation of the EGR-1 sites reduced but did not abolish induction by serum or PMA, suggesting that this residual activity represented the immediate early component. Further mutation of the Sp1 sites completely abolished induction. Sp1 was constitutively expressed by HeLa cells, consistent with a proposed role in immediate early induction. However, induction of the TF promoter may be directly or indirectly mediated by Sp1. The transcriptional activity of Sp1 may be increased by some form of post-translational modification, such as phosphorylation, although our Western blots did not detect a change in the ratio of the 95- and 105-kDa forms of Sp1. Nevertheless, we cannot exclude the possibility that PMA and serum induce phosphorylation of Sp1 without detectable changes in electrophoretic mobility. Alternatively, PMA and serum may activate pre-existing coactivators that interact with both Sp1 bound to the SRR and the basal transcriptional machinery to mediate transcriptional activation (41). Another possible explanation for these results is that another as yet unidentified transcription factor is required for the immediate early induction of the TF gene. This protein may bind to the SRR at a site(s) distinct from the 12-bp motifs and possibly interact with Sp1 to mediate activation of the TF gene.

The delayed early component of TF gene induction required EGR-1 sites. Mutation of the EGR-1 sites reduced PMA and serum induction of the TF promoter. Moreover, the wild-type TF promoter, but not plasmids containing mutations in the EGR-1 sites, was transactivated by over-expression of EGR-1 in quiescent HeLa cells. Induction of TF mRNA is maximal 2 h after PMA or serum stimulation (9) and is delayed compared...
with c-fos, egr-1, and other immediate early genes. This delayed induction is consistent with a role for newly synthesized EGR-1 in the regulation of the TF promoter. Previously, we demonstrated that TF mRNA was induced in Hela cells in the presence of the protein synthesis inhibitor, cycloheximide (9). However, cycloheximide can both induce TF gene transcription and stabilize TF mRNA (42), which could compensate for the loss of the delayed early component due to inhibition of de novo synthesis of EGR-1. Recently, it was demonstrated that transcriptional activation of nurr77, which was also originally identified as a serum-inducible immediate early gene, is mediated by both an immediate early component and an EGR-1-dependent delayed early component (43). In addition, EGR-1 may be involved in the regulation of the c-myc gene, which contains an EGR-1 site (17) and also exhibits delayed induction (4). Thus, the rapid expression of EGR-1 in serum and PMA stimulated cells may contribute to the transcriptional activation of a several genes previously classified as immediate early genes. Transcriptional activation of the TF gene required the Sp1 and EGR-1 sites because mutation of all six sites within the SRR abolished both PMA and serum induction. However, several differences were noted between PMA and serum. For instance, serum was a less potent inducer of the wild-type TF promoter in this vector system than PMA, which may be due, in part, to the relative levels of EGR-1 induced by these agonists. In addition, PMA stimulation phosphorylates EGR-1 more efficiently than serum stimulation, and highly phosphorylated forms of EGR-1 bind to DNA with increased affinity (36) and may have increased transcriptional activity. Mutation of the Sp1 sites reduced serum induction but increased PMA induction. This would abolish Sp1 competition for the 12-bp motifs, again suggesting that EGR-1 induced by PMA may be a more potent transcriptional activator than EGR-1 induced by serum. These differences may reflect activation of different signaling pathways. Previously, we showed that the signaling mechanisms for serum and PMA stimulation are distinct (9). TF mRNA induction by both agonists required intracellular calcium mobilization, whereas inhibition of protein kinase C abolished induction of the TF gene by PMA but had no effect on induction by serum (9). Nevertheless, our data showed that the same three 12-bp motifs within the SRR mediated PMA and serum induction of the TF promoter.

Comparison of the DNA sequences of the human, murine, and porcine TF promoters is shown in Fig. 7. The Sp1 and EGR-1 sites in regions 1, 2, and 3 are indicated. The Sp1 site in the 12-bp motif in region 1 is completely conserved, whereas the 5’ triplex of the EGR-1 site is not conserved. The consensus EGR-1 sites in the 12-bp motifs in region 2 from the human and porcine TF promoters contain a single nucleotide substitution in the murine TF promoter. The porcine TF promoter did not contain the 12-bp motif in region 3, which is consistent with our previous results indicating that two of the three 12-bp motifs within the human promoter are sufficient for PMA and serum induction (9). The spacing of the two 12-bp motifs in regions 1 and 2 is precisely conserved in all three TF promoters, suggesting that protein–protein interactions may be important in the assembly of transcription factors on this region.

Our data suggest a model for PMA and serum induction of the TF gene in which Sp1 is constitutively expressed by uninduced cells and binds to all three sites within the promoter to regulate basal expression of the TF gene. We speculate that in PMA- or serum-stimulated cells Sp1 is either post-translationally modified or interacts with pre-existing, activated coactivators to mediate immediate early induction of the TF gene in the absence of de novo protein synthesis. Alternatively, other members of the Sp1 multigene family may be involved in TF gene regulation (25, 26). For instance, Sp3 binds to a GC box and is present at low levels in Hela cells (44). However, Sp3 represses Sp1-mediated activation via competition for Sp1 binding sites (44). In stimulated Hela cells, the ability of Sp1 and Sp3 to bind DNA may be changed in favor of Sp1, permitting immediate early activation of the TF gene. In addition, stimulation of the cells rapidly induces expression of EGR-1, which binds to the three EGR-1 sites in the SRR to mediate a delayed early response and achieve maximal transcriptional activation of the TF gene. The ability of EGR-1 to compete Sp1 binding to the 12-bp motifs within the SRR may be determined by both the levels of EGR-1 protein and their degree of phosphorylation. Thus, these overlapping EGR-1/Sp1 sites within the three 12-bp motifs may function to control the level and pattern of TF expression in different cell types exposed to a variety of agonists.

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