A Novel ets-related Transcription Factor, ERT/ESX/ES-E-1, Regulates Expression of the Transforming Growth Factor-β Type II Receptor

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Shin-Geon Choi, Youngsuk Yi, Yong-Seok Kim, Mariko Kato, Jay Chang, Hwan-Wook Chung, Ki-Baik Hahm, Han-Kwang Yang, Horace H. Rhee, Yung-Jue Bang‡, and Seong-Jin Kim§

From the Laboratory of Cell Regulation and Carcinogenesis, NCI, National Institutes of Health, Bethesda, Maryland 20892-5055, and the iCancer Research Center, Seoul National University College of Medicine, Seoul, Korea

A 2.5-kilobase cDNA clone that encodes a 371-amino acid novel transcription factor was isolated from a human placenta cDNA library using a yeast one-hybrid system. The novel ets-related transcription factor (ERT) showed a homology with the ETS DNA-binding domain. Using constructs of the transforming growth factor-β (TGF-β) type II receptor (RII) promoter linked to the luciferase gene, we have demonstrated that ERT activates transcription of the TGF-β RII gene through the 5′-TTTCTGTTTCC-3′ response element spanning nucleotides +13 to +24 and multiple additional ETS binding sites between −1816 and −82 of the TGF-β RII promoter. A specific interaction between ERT and the ETS binding sites was also demonstrated using an electrophoretic mobility shift assay. Deletion mapping of ERT protein suggests that the transactivation domain resides in the amino terminus while the DNA-binding domain is localized to the carboxyl-terminal region. Our results suggest that ERT might be a major transcription factor involved in the transcriptional regulation of the TGF-β RII gene.

Transforming growth factor-β (TGF-β) plays a critical role in many cellular processes, including regulation of the cell cycle, cell differentiation, and extracellular matrix synthesis (1, 2). Aberrant TGF-β function has been implicated in the pathogenesis of many diseases, and it has also been suggested that diminished responsiveness to TGF-β may contribute to the process of malignant transformation (1). This decreased responsiveness to TGF-β could be caused by defects not only in TGF-β expression or activation but also by defects in the regulation of TGF-β receptors (3–6).

Much work has recently been directed toward characterizing the TGF-β receptors and their intracellular signaling pathways. TGF-β type II and type I receptors (TGF-β RII and RI, respectively) are transmembrane serine/threonine kinases that together are sufficient for signal transduction (7). Association between the type I and type II receptors is essential for signaling responses (8). It has been repeatedly demonstrated that a genetic alteration of either RI or RII resulting in dominant negative or loss of function can lead to loss of responsiveness (3–6, 9–12).

In a previous study, our laboratory described a series of gastric cancer cell lines in which resistance to TGF-β is correlated with gross structural mutations in the TGF-β RII gene (3). We have now studied several additional TGF-β-resistant cell lines in which Southern analysis failed to show gross deletions or rearrangements, yet in which no TGF-β RII protein or mRNA was produced. This suggested that abnormalities in transcriptional regulation of the type II receptor might also be found to underlie certain instances of escape from TGF-β-mediated growth inhibition.

We have recently cloned and sequenced the promoter region of the TGF-β RII gene, identified several positive and negative transcriptional regulatory elements, and reported the relevant target sequences for three putative novel transcription factor complexes (3, 14). Basal levels of transcription are determined by the core promoter element in cooperation with both PRE1 and PRE2 (positive regulatory elements 1 and 2). PRE1, consisting of nucleotides −219 to −172, contains two discrete target sequences that bind an API/CREB-like transcription factor in addition to an unidentified novel transcription factor complex. PRE2 is located between +1 and +55 and contains two overlapping target sequences, both of which appear to bind novel transcription factor complexes.

To identify potential transcriptional activators of the TGF-β RII gene, we adapted the yeast one-hybrid system (15) to find proteins that recognize the PRE2 of the TGF-β type II receptor gene. Screening a human placenta cDNA library fused to the GAL4 activation domain, we isolated a cDNA clone that induced greater LacZ activity. DNA sequencing analysis of a corresponding plasmid, pACT2ERT, revealed that the encoded gene belongs to a novel member of the ets transcription factor family (16–22). Comparison of the nucleotide sequence of ERT to the recently reported epithelial specific ets-family member, ESX/ES-E-1 (23, 24) showed it to be identical, but the ERT cDNA revealed an additional 524 nucleotides in the 3′-UTR. Further, we demonstrate that the ERT protein specifically binds to the PRE2 region of the TGF-β RII gene and activates its transcription.

MATERIALS AND METHODS

Reporter Constructs for Library Screen—The following oligonucleotides, 5′-GAGGAGTTCCTGTGTTTTCCCCGCC-3′ and 5′-GGCGGG-GAAAAGCTGAAGTCCCT-3′, containing the previously described PRE2 binding site were synthesized and annealed (13). The PRE2 mutant oligonucleotides were constructed by replacing the underlined sequences with 5′-AAGTG-3′ and 5′-CACCT-3′, respectively. The oligo...
nucleotides were ligated and subcloned into the BamHI site of pUC18. A fragment corresponding to a four tandem repeat was subcloned into the yeast reporter plasmids, pHISi and pLacZi (CLONTECH). The reporter constructs were subsequently integrated into the yeast strain YM4271 yielding YM4271::PRE2::His3 (or lacZ) and YM4271::PRE2M::His3 (or lacZ). These yeast strains were used as host strains for the library screen.

**Bacterial and Yeast Strains**—Saccharomyces cerevisiae YM4271 (MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-903, tyr1-501) was purchased from CLONTECH and used for yeast transformation. Escherichia coli strains DH5α and DH10B (Life Technologies, Inc.) were used for subcloning and electroporation experiments.

**Screening of the cDNA Library**—The histidine yeast reporter strain YM4271::PRE2::His3 was transformed with a MATCHMAKER human placenta cDNA library (CLONTECH) by the LiAc/polyethylene glycol method. Approximately 5 × 10⁶ transformants were plated per 150-mm dish containing his’ minimal selective medium supplemented with 45 mM 3-aminotriazole. Approximately 2 × 10⁶ cDNA plasmids were screened in three different transformations. Based on large colony size and rapid growth, a total of 30 histidine positive clones were selected. Plasmids were recovered and electroporated into the E. coli strain DH10B. Plasmids were rescreened by transforming YM4271::PRE2::lacZ and plated on leu’ ura’ minimal medium with 45 mM 3-aminotriazole. The filter replica method using X-gal (40 μg/ml) was used to confirm β-galactosidase activities. One plasmid, pACT2ERT, showed the strongest blue color. The specific DNA binding of pACT2ERT was confirmed by comparing the β-galactosidase activities of wild-type and mutant reporter strains using both the filter replica method and O-nitrophenyl β-D-galactopyranoside (ONPG) liquid method (CLONTECH).

**Plasmid Constructions**—The plasmid pcDNA3.1-ERT was generated by subcloning a 2.5-kb EcoRI-HindIII fragment containing the entire ERT coding sequence into the EcoRI-HindIII sites of pcDNA3.1(−) (Invitrogen). The plasmid pG3L-pro derivatives including wild-type and mutant PRE2 sequences were constructed by inserting the KpnI-XbaI fragment of pUC18::PRE2 and pUC18::PRE2M into the KpnI-NheI site of pG3L-pro (Promega). The construction of plasmid 219+3’TBP-II-luc is explained elsewhere (13). TGF-β RII promoter-luciferase constructs were generated by polymerase chain amplification using genomic DNA containing the 5’-untranslated region of TGF-β RII as a template. Amplified DNA fragments were cloned into a promoterless luciferase expression plasmid, pGL2 (Promega) using BglII and SalI restriction sites built into the oligonucleotides used for amplification. The sequences of the polymerase chain reaction-generated portions of all constructs were verified by DNA sequencing.

Synthetic wild-type interleukin-2 receptor β-chain (25), β2 integrin CD18 (26), HTLV-ILTR (27), HIV-2 LTR (28), polyomavirus PEA3 (29), and T-cell receptor a (TCRα) enhancer Tox (30) ets site oligonucleotides containing HindIII and SalI ends were inserted into the HindIII/SalI sites of the pGL2-promoter plasmid (Promega). All GAL4-ERT fusion plasmids were constructed by inserting the appropriate ERT DNA fragment in-frame to the GAL4 (1–147) sequence in the vector pSG424 (31). ERT DNA fragments were produced by polymerase chain reaction. For the construction of AU-tagged ERT expression plasmids, polymerase chain reaction products were ligated into elongation factor-1 promoter, pCEFL-AU5, using standard methods to generate pCEFL-AU-ERT (1–371, 1–301, 1–200, 1–120, 120–371, 200–371, and 301–371).

**In Vitro Transcription and Translation**—One μg of DNA of pcDNA3.1(−)-ERT constructs was used as the DNA template for in vitro RNA transcription using T7 RNA polymerase (Promega). The RNAs were translated in vitro using rabbit reticulocyte lysate (Promega) and [35S]methionine. Expressed proteins were electrophoresed on a 4–20% SDS-polyacrylamide gradient gel, dried, and autoradiographed.

**Electrophoretic Mobility Shift Assay (EMSA)**—In vitro translated protein-DNA complexes were formed by incubating at room temperature for 20 min with 10,000 cpm of 32P-labeled probe, 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, bovine serum albumin (300 μg/ml) in 20 μl of binding mixture.

**Transient Transfection and Luciferase Assays**—HepG2 human hepatoblastoma cell line was maintained in minimal essential medium supplemented with 10% fetal bovine serum. For the transient expression assays, cells were transfected using the Lipofectin-mediated transfection method (Life Technologies, Inc.). Following incubation with Lipofectin for 15 min, cells were incubated for 48 h. The cells were then harvested, and luciferase activity was measured. β-Galactosidase activity was used to correct for transfection efficiencies.

**Northern Blot analysis**—SNU620 human gastric cancer cells, HeLa229 human cervical cancer cells, and SK-BR3 breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Total RNA was isolated from cells using guanidium...
codes a 371-amino acid protein with a predicted molecular mass of 41,000 daltons. Comparison of the nucleotide sequence of ERT to the recently reported epithelial specific ets-family member, ESX/ESE-1 (23, 24), was identical, but the ERT cDNA revealed an additional 524 nucleotides in the 3′-UTR. Comparison of the amino acid sequences of ERT and other ets family members revealed a high degree of homology in the ETS domain, located at the carboxyl-terminal region of the molecule. Approximately 40% of the ETS domain amino acid sequence is shared by ERT and other ets family members such as ets-1 (16), ets-2 (16), ERG (17), Spi-1 (18), E1A-F (19), ER81 (20), and ERM (21) in the ETS domain. There is no significant homology between ERT and any other ets family members outside the carboxyl-terminal domain.

Northern blot analysis of various cancer cell lines showed a major 2.2-kb and a minor 2.7-kb message when probed with the cloned 2.5-kb ERT cDNA insert (Fig. 2). Since the major 2.2-kb ERT mRNA is smaller than the cloned 2.5-kb ERT cDNA insert, we probed the same blot with the 3′-UTR sequences from nucleotides 1980 to 2529. This 3′-UTR probe recognized only the 2.7-kb ERT transcript (Fig. 2, lanes 4–6), suggesting that the two different ERT transcripts resulted from alternative poly(A) signals.

Transcriptional Activation of the TGF-β RII Promoter by ERT—Most of the ets family members are known to be potent transcriptional activators when tested in transient transfection assays (22). To analyze the ability of ERT to activate the TGF-β type II receptor promoter, the TGF-β RII promoter-luciferase constructs were cotransfected with an expression vector for ERT into HepG2 human hepatoblastoma cells. While ERT did not induce luciferase expression in the control reporter construct, we found ERT to be a potent transcriptional activator of the TGF-β type II receptor promoter construct. As seen in Fig. 3, –1,670/+36pTBPII-luc construct was induced 5-fold by ERT. The –1,670/+36mpTBPII-luc construct, in which the second positive regulatory element was mutated, was also activated more than 2.5-fold, suggesting that the fragment between –1,670 and +2 contains multiple ERT regulatory sequences (Fig. 3). In the previous study (13), we reported that at least two distinct nuclear DNA binding proteins shared a common recognition sequence from +11 to +29 in the PRE2 of the TGF-β type II receptor promoter. This sequence contains two putative target sequences for protein-binding of ets family members in a reverse-orientation (5′-GGAAACAGGAAA-3′). Competitive inhibition for DNA binding to the +1/50 sequence was abolished by mutation of nucleotides +16 to +20 (see Fig. 6B).

To characterize the putative ERT response element in the second positive regulatory element, we generated chimeric constructs containing four tandemly linked copies of the PRE2 sequence of the TGF-β RII promoter between +1 and +36 linked to a pGL2-promoter. Mutated forms of the putative ERT responsive elements (shown in bold) were generated by nucleotide substitution as described in Fig. 4. In cotransfection assays, ERT induced luciferase expression by the wild-type construct (WT-luc) approximately 3.5-fold over that of the control. Mutation of nucleotides +16 to +20 of the ERT response element in construct MT1-luc resulted in significant reduction in transcriptional activation (Fig. 4B). Therefore, the sequence 5′-TTTCTCTGTTTTCC-3′ (GGAAACAGGAAA in a reverse orientation) located between +13 and +24 appears to be responsible for ERT-mediated activation of the TGF-β type II receptor gene. Next, we mutated the individual ETS binding site (EBS) in the second positive regulatory element to see whether both EBSs are required for the maximum induction of TGF-β type II receptor promoter activity by ERT. MT2, in which the first EBS...
is mutated (Fig. 4A), was induced 2-fold by ERT, and MT3, in which the second EBS was mutated, was also induced 1.5-fold, whereas MT4, in which both EBSs were mutated, showed little induction (Fig. 4B). These results suggest that ERT can activate the TGF-β type II receptor promoter through either EBS but that both sites are required for the maximal induction.

We next examined whether additional EBS might be identified in the entire TGF-β RII promoter. A computer search revealed multiple copies of EBS between −1816 and +25 (Fig. 5). As seen in Fig. 3, the activity of TGF-β type II receptor-luciferase constructs was induced 2- to 4-fold by ERT.

The activity of construct −70/+36 was induced 2-fold by ERT, suggesting that this induction was due to EBSs located in +8/+36 promoter region since no EBS is present in the sequences between −70/−1. Upon mutating the two EBSs between +8/+25 (−1670/+36mt), basal levels of activity dropped 2-fold suggesting that these sites are required for the basal expression of the TGF-β type II receptor. In the presence of
ERT, the mutated construct induced activity 2-fold, indicating that other upstream EBSs are also responsive to ERT.

**ERT Binds to the PRE2 of the TGF-β RII Promoter**—To analyze the ability of ERT to bind to the TGF-β RII promoter element PRE2, in vitro translated ERT protein was made by subcloning the EcoRI-BglII portion of the insert into the EcoRI-BamHI site of pcDNA3.1. This 41-kDa in vitro translated ERT protein was consistent with the predicted size of the open reading frame and was used for the electrophoretic mobility shift assay (Fig. 6A). A radiolabeled PRE2 (+1/+50) probe was incubated with in vitro translated ERT protein in competition with unlabeled PRE2 and mutant PRE2 oligonucleotides. While specific unlabeled competitors competed for labeled protein-DNA complexes (Fig. 6B, lane 4), oligonucleotides mutated in nucleotides +16 to +20 comprising the ETS-binding site (lane 5) did not compete for binding to ERT. Deletion of the carboxyl-terminal ETS-domain from the ERT protein produced a smaller protein of approximately 30 kDa (Fig. 6A, lane 3) that failed to interact with the wtPRE2 (Fig. 6B, lane 6).

**ERT Binds to Functionally Important ETS-related Binding Sites in a Variety of Genes**—To analyze the DNA sequence requirements for the binding of ERT and the relative binding affinities, we designed oligonucleotides encoding a whole spectrum of different functionally relevant binding sites for ets-related factors, including the site in the interleukin-2 receptor β-chain gene (Fig. 7A). The relative binding affinities of ERT for these sites were compared with its affinity for the human TGF-β RII promoter ETS sites. Equivalent amounts of wild-type labeled oligonucleotides were used as probes in EMSAs with equal amounts of full-length ERT in vitro translated protein (Fig. 7B). The in vitro translated ERT formed a complex with all the ets-related binding site oligonucleotides tested but with different affinities (Fig. 7B). EBS oligonucleotides in the TCRα enhancer Tα2, polyomavirus PEA3, and HTLV-1 LTR promoters bound to the ERT with strong affinity, whereas ERT interacted with IL-2-receptor β2 chain, CD18, and HIV2 LTR ets binding site oligonucleotides with weaker affinities. To determine if ERT binding correlates to the transcriptional induction by ERT, we cotransfected HepG2 cells with these oligonucleotides linked to a luciferase reporter gene and an ERT expression vector or a control pGL2-pro. A direct correlation was found between the relative strengths of the binding affinities and the level of activity induced by ERT (Fig. 7C). PEA3 was induced 5-fold, Tα and HTLV-1 were both induced 3-fold, while the other weak ERT affinity binding oligonucleotides showed no appreciable induction, suggesting that binding is necessary for ERT-mediated transcriptional induction.

**Mapping of Transactivation Domain**—Transcription factors often display a modular structure with domains being responsible for DNA binding, transactivation, or protein-protein interaction. To identify potential transactivation domains within...
ERT, several truncations of ERT were tested in transient transfection assays (Fig. 8). Deletion of the first 200 amino acids led to a severe reduction of ERT-mediated transcription, implicating the existence of an amino-terminal transactivation domain. Interestingly, the ERT DNA binding domain alone (200/371 or 301/371) suppressed basal activity of TGF-β type II receptor promoter, suggesting that it acts as a dominant negative mutant.

To analyze the location of the transactivation domain in ERT, fusions of ERT amino acids to the DNA binding domain of the yeast transcription factor GAL4 were constructed. These plasmids were transfected into the HepG2 cells with a luciferase reporter (G5BLuc) containing five GAL4 binding sites upstream from the E1B TATA box (Fig. 9). Comparable expression of the GAL4-ERT fusion proteins was investigated by Western blot analyses using anti-GAL4 antibodies (data not shown). Significant increases of basal transcription were observed with the GAL4-ERT-(1–371). Transcriptional activity was diminished with deletion of the amino terminal region of ERT (Fig. 9B). Basal level of transcription was significantly elevated with the GAL4-ERT-(1–200) fusion protein. In addition, the GAL4-ERT-(1–300) is less active than GAL4-ERT-(1–200), suggesting that amino acids 200 to 300 exert an inhibitory effect on transactivation (Fig. 9B). These data are in agreement with the identification of a transactivation domain at the amino terminus of ERT.

**DISCUSSION**

We have isolated a new member of the ets family of transcription factors, which is a potent transcriptional activator of the TGF-β RII gene, by using a novel genetic approach based on the yeast one-hybrid system. Using this genetic selection to screen a human placenta cDNA library for sequences encoding DNA-binding domains that can recognize the PRE2 of the human TGF-β RII gene in yeast, we isolated a human cDNA that codes for a protein, ERT, that binds to the PRE2 of the TGF-β RII promoter in a sequence-specific manner and activates transcription. The deduced amino acid sequence shows high homology with the ETS-domain, the DNA binding region in the ets family genes. Comparison of the nucleotide sequence of ERT to the recently reported epithelial specific ets-family member, ESX/ESE-1 (23, 24) was identical, but the ERT cDNA revealed an additional 524 nucleotides in the 3’-UTR.

In a previous study, we demonstrated that PRE2 contained at least one nuclear protein recognition sequence from +11 to +29 (13). This region contains two direct repeats of the purine-rich sequences (GGAAAC) in a reverse-orientation. Competition for binding to ERT was abolished by mutation of this sequence, suggesting that these purine-rich sequences are the binding sites for ERT. Expression of exogenous ERT increases the level of transcription from the TGF-β RII promoter, implying an activating role for ERT in TGF-β RII expression. We
have detected high-affinity binding sites for ERT in the regulatory regions of various genes, and we have demonstrated that ERT can transactivate the isolated ETS sites of these promoters.

Proteins of the ets gene family members have a conserved DNA-binding domain (the ETS domain) and regulate transcriptional initiation from a variety of cellular and viral gene promoters and enhancer elements, including the human interleukin-2 receptor β-chain gene promoter (25) and the human β2 integrin CD18 promoter (26). A combination of EMSA and methylation interference studies on the binding of ETS proteins to the target sequences has shown that the GGAA purine core is essential for the specific binding of ETS-related proteins. All members of the ets family share a common recognition sequence, whereas the flanking sequences are divergent for different members of the ets family. Differences in ERT
binding to and ERT-mediated transactivation of a variety of genes containing EBS examined in this study could therefore reflect the precise recognition sequence for ERT. We identified multiple EBS-like sequences in the region between −1816 and +36 by a computer analysis. The TGF-β RII promoter PRE2 contains two copies of the consensus core sequence GGAA located at +14 to +17 and +21 to +24 which are between +1 and +50. This region is essential for TGF-β RII promoter and enhancer activities. It is noteworthy, however, that these ETS-binding sites by themselves are not sufficient to fully activate the promoter since mutational analysis reveals that the first positive regulatory element (PRE1) of the TGF-β RII promoter cooperates with the second positive regulatory element (PRE2) to sustain basal levels of promoter activity (13). Since the sequence between −1 and −1883 is also responsive to ERT, it is possible that these ETS-binding sites functionally interact with the first positive regulatory element to achieve the full promoter activity of the TGF-β RII gene.

The expression patterns of different members of the ets gene family vary between tissues (22). Many members of this family are expressed in hematopoietic cells, suggesting a role for these members of the ets family in hematopoietic cell growth and differentiation (22). All the ets family genes, with the exception of yan (32) and ERF (33), are known to be potent transcriptional transactivators. Recently, Chang et al. (23) and Oetttgen et al. (24) reported the identification of a new epithelium-restricted ETS, ESX/ESX-1, based on a search of expressed sequence tags. The sequence of the ESX gene is identical to the ERT nucleotide sequence; however, our ERT cDNA is 524 nucleotides longer in the 3′-untranslated region and 150 nucleotides longer in the 5′-untranslated region compared with the published ESX sequence (23, 24). Our preliminary results show that other ets family members such as ETS-1 and ETS-2 also induce TGF-β RII promoter activity, suggesting that ets family members may be one of the major transcription factors involved in regulation of TGF-β RII gene expression. Since most cells express the TGF-β receptors, it is possible that expression of the TGF-β RII gene may be regulated by distinct ets family members in different tissues.

TGF-β plays a critical role in many cellular processes, including regulation of cell cycle and cell differentiation, and it has now been demonstrated that aberrant expression of TGF-β receptors may play a role in a wide variety of human pathologies. We have recently identified a subset of human gastric cancer cell lines that are resistant to TGF-β and that lack TGF-β RII mRNA expression despite evidence of a normal gene, suggesting that transcriptional regulation may play an important role in controlling TGF-β RII expression. We demonstrate that there is a strong correlation between expression patterns of TGF-β RII mRNA and ERT mRNA in human gastric cancer cell lines. Examination of the mechanisms underlying loss of TGF-β RII expression in different human gastric cancer cell lines suggests a complex interplay between mutational events and transcriptional regulation. Given the importance of the ets family genes in regulating TGF-β RII expression, it is quite likely that regulation of this family of transcription factors, including ERT, will emerge as a key mechanism controlling cellular responsiveness to TGF-β.

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