Identification of Ets-1 as an Important Transcriptional Activator of CTP:Phosphocholine Cytidylyltransferase in COS-7 Cells and Co-Activation with Transcriptional Enhancer Factor-4*

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Running Title: Ets-1 enhances CT enzymatic transcription
Phosphatidylcholine biosynthesis via the CDP-choline pathway is primarily regulated by CTP:phosphocholine cytidylyltransferase (CT). Transcriptional enhancer factor-4 (TEF-4) enhances the transcription of CTα in COS-7 cells by interactions with the basal transcription machinery (Sugimoto, H., Bakovic, M., Yamashita, S., and Vance, D.E. (2001) J. Biol. Chem. 276, 12338-12344). To identify the most important transcription factor involved in basal CTα transcription, we made CTα promoter-deletion and -mutated constructs linked to a luciferase reporter and transfected them into COS-7 cells. The results indicate that an important site regulating basal CTα transcription is -53/-47 (GACTTCC), which is a putative consensus binding site of Ets transcription factors (GGAA) in the opposite orientation. Gel shift analyses indicated the existence of a binding protein for -53/-47 (GACTTCC) in nuclear extracts of COS-7 cells. When anti-Ets-1 antibody was incubated with the probe in gel shift analyses, the intensity of the binding protein was decreased. The binding of endogenous Ets-1 to the promoter probe was increased when TEF-4 was expressed, however, the amount of Ets-1 detected by immunoblotting was unchanged. When cells were transfected with Ets-1 cDNA, the luciferase activity of CTα promoter constructs was greatly enhanced. Co-transfection experiments with Ets-1 and TEF-4 showed enhanced expression of reporter constructs as well as CTα mRNA. These results suggest that Ets-1 is an important transcriptional activator of the CTα gene and that Ets-1 activity is enhanced by TEF-4.
Phosphatidylcholine (PC) is the major membrane phospholipid in mammalian cells and tissues. PC is made in all nucleated cells via the CDP-choline pathway in which CTP:phosphocholine cytidylyltransferase (CT) is recognized as an important rate-limiting and regulated enzyme (1-4). The first mammalian CT was purified from rat liver (5, 6), and the corresponding CT cDNA was cloned and expressed (7). CT is ubiquitous and the most active form of CT (8,9) although cDNAs for two other isoenzymes, CTβ1 (8) and its splice variant CTβ2 (9), were recently cloned, and are encoded by a second gene. All isoforms contain a highly conserved catalytic domain and a helical lipid-binding domain (8). CT and CTβ2 contain a highly phosphorylated domain at their carboxyl termini (1-4, 9-11), while CTβ1 lacks this domain (8). The functions of the individual domains for regulation of CT activity have been extensively studied. The helical domain for the binding of specific lipids (1-4, 12, 13), and a highly phosphorylated domain at the carboxyl terminus (1-4, 11, 14-17), are important for the modulation of CT activity. However, less is known about the regulation or function of CTβ1 and CTβ2. Zhang et al. showed that CTβ2 is induced in macrophages from CTβ-deficient mice and a low level of CTβ2 activity is apparently enough to keep the cells viable (18).

In addition to post-translational regulation, CT mRNA levels have been reported to be regulated transcriptionally and post-transcriptionally. Tessner et al. (19) provided the first evidence for increased CT mRNA in colony stimulating factor 1-stimulated macrophages. Houweling et al. (20) demonstrated that CT is regulated at the level of its mRNA in rat liver after partial hepatectomy. The maximal increase in the CT mRNA coincided with maximal DNA synthesis 24 h after partial hepatectomy (21). CT is highly expressed during the perinatal period and the expression of CT is positively associated with hepatic cell division (22,23). Golfman et al. (24) recently showed that CT mRNA increased during the S phase of the cell cycle in C3H10T1/2 cells. Several reports indicate that CT can be
regulated post-transcriptionally, apparently by reduction in the rate of mRNA degradation (19,25). However, the precise mechanisms involved in regulating CT mRNA stability still have to be elucidated.

Tang et al. (26) isolated the murine CT[] gene (Ctpct) and showed that the exon/intron organization of the gene closely resembles the functional domains of the enzyme. The gene is transcribed from two transcriptional start sites, lacks a TATA box, but contains GC-rich regions. The 5'-terminal ~ 200 bp of the proximal promoter appears to contain Sp1, NF-κB, Ets, and cAMP responsive element binding protein (CREBP) binding sites as shown in Fig. 1. Bakovic et al. (27) characterized its regulatory elements and associated factors. Three Sp1-binding regions (-31/-9, -88/-50, and -148/-128) have basal, activator and suppressor promoter activities, respectively. Sp1, Sp2, and Sp3 can competitively bind to these regions and the relative abundance of these factors regulates promoter activity of the CT[] gene (28). More recently we have shown that transcriptional enhancer factor-4 (TEF-4) binds to the promoter region between -97 and -89 as shown in Fig. 1 and stimulates the expression of the CT[] gene by association with the basal transcription machinery (29). TEF-4 is closely related to TEF-1 (30, 31) that was previously isolated as a regulatory protein of the SV40 enhancer (32). Kast et al. (33) and Mallampalli et al. (34) reported a functional sterol response element 156 bp upstream of the transcriptional start site. Induction of CT mRNA was observed when Chinese hamster ovary cells and alveolar type II epithelial cells were cultured in lipoprotein-deficient serum, respectively. However, the physiological importance of this sterol response site in transcriptional regulation of the CT[] gene is not clear (35).

In this study, we provide evidence that Ets-1 is a major transcription factor involved in the basal transcription of the CT[] gene. Ets-1 was first reported as a proto-oncogene for avian erythroblastosis (36).
EXPERIMENTAL PROCEDURES

Materials - The luciferase vector, pGL3-basic, that contains the cDNA for Photinus pyraeus luciferase, the control pRL-CMV vector that contains the cDNA for Renilla reniformis luciferase, and the Dual-luciferase Reporter Assay System were obtained from Promega (Madison, WI). FuGENETM6 transfection reagents, Dulbecco’s modified Eagle’s medium (high glucose), and fetal bovine serum were from Roche Molecular Biochemicals (Indianapolis, IN), Sigma (Saint Louis, MO), and Life Technologies, Inc. (Rockville, MD), respectively. COS-7 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Preparation of Deleted and Mutated CTca Promoter-Luciferase Reporters - Various 5'-deleted CTca promoter regions, LUC.C7 (-1268/+38), LUC.C8 (-201/+38), LUC.D1 (-90/+38), LUC.D2 (-130/+38), LUC.D3 (-52/+38), LUC.D4 (-10/+38), and LUC.D5 (+10/+38), inserted into the promoter-less luciferase vector pGL3-basic (Promega) were prepared as described previously (27). LUC.D3.25 (-43/+38), LUC.D3.5 (-31/+38), and LUC.D4.5 (-71/+38) clones were obtained from LUC.C8 by PCR using the corresponding forward primers, 5'-CAGGTACCCAGTCCGGTCAGATGTTTCCGG-3', 5'-CAGGTACCCAGATGTTTCCCGGGCGTCTCC-3', and 5'-CAGGTACCAGGGCGGGCGGGACT-3', respectively, and GL Primer 2 (Promega) as a universal reverse primer from the luciferase vector. The amplified fragments were purified, cut with KpnI/HindIII and cloned into the corresponding site of the pGL3-basic vector. Fig. 1 shows the partial structure of the mouse Ctppct promoter from -212 to +38, and indicates the start positions of the deletion mutants. When we searched the CTca promoter region by TRANSFAC transcription factor database, several important consensus elements for the binding of transcription factors were identified (Fig. 1). To prepare mutated promoters, GCCC (-139/-136)
was mutated to AGCT and named LUC.mSp1(1)/C7 or D1.5, CGGGCG (-67/-62) was changed to AATTCA and named LUC.mSp1(2)/C7 or D1.5, GCGGG (-58/-54) was mutated to AACAA and named LUC.mNF-κB/C7 or D1.5, GACTTC (-53/-48) was converted to ACCAAA and named LUC.mEts-a/C7 or D1.5, and TGAC (+20/+23) was changed to AAA and named LUC.mEts-b/C7 or D1.5, and TGAC (+20/+23) was changed to AAA and named LUC.mCREB/C7 or D1.5.

These mutations were created from LUC.C7 or LUC.D1.5 by PCR using QuickChange™ site-directed mutagenesis kits (Stratagene, La Jolla, CA). The mutated complementary primers were used for making each mutated construct and the resulting plasmids were used for transformation of JM109 cells. The amplified plasmids were cut with KpnI/HindIII and cloned into the corresponding site of the pGL3-basic vector. All deleted and mutated constructs were sequenced and correct ones were selected for further experiments.

Construction of TEF-4 and Ets-1 Expression Vectors- The mouse TEF-4 expression vector carrying the cDNA of TEF-4 was obtained by the yeast one-hybrid system as reported (29). The mouse full-length cDNA coding murine Ets-1 was obtained through PCR of a reverse-transcribed product of mRNA from NIH cells using Superscript II (Invitrogen, Carlsbad, CA). PCR was performed with the complementary primers, 5’-GGCACCATGAAGGCGGCCGTCGATC-3’, and 5’-GTCAGCATCCGGCTTTACATCCAGC-3’ with Takara Ex Taq™ DNA polymerase (Takara-Bio. Co., Tokyo, Japan). The 1.4 kb PCR product was cloned into pcDNA3.1/V5 (Invitrogen) in frame with V5-tag, and the sequence was confirmed.

Tissue Culture, Transfection, and Luciferase Assays- COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells (1 x 10⁵) were plated on 35-mm plates (Falcon-Becton Dickson Labware, Franklin Lakes, NJ) and grown overnight. Three µL of FuGENE™6 was suspended in 100 µl of serum-free medium and mixed with 0.5 µg of the
CT promoter-luciferase constructs (see above), 0.001 µg of pRL-CMV *Renilla* vector as a transfection control, and 0.5 µg of either pcTEF-4 or pcDNA (Invitrogen). Transfection was initiated by dropwise addition of DNA suspension to the cell culture. Forty-eight h later cells were harvested, lysed in 200 µl of Passive lysis buffer (Promega), and 10 µl of the cell lysate was used for the dual-luciferase assay according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency by using the ratio of the activities obtained with the CT promoter deletion constructs (see above) and the pRL-CMV construct carrying the cytomegalovirus promoter-luciferase fusion. When we assayed luciferase activities with Ets-1, pcDNA was used so that the plasmid content in each experiment was equal, and luciferase values were normalized against total protein concentrations determined by protein assay (Bio-Rad, Hercules, CA).

**Preparation of Nuclear Extracts and Electromobility Gel-shift Assays**- Nuclear extracts from COS-7 cells were prepared according to Andrews and Faller (37) with minor modifications (27). Five hundred pmol of the opposite strands of pEts (5’-GGCGGGAGGCAGGACTCTCCGGTCCGCAGTC-3’), pmEts-a (5’-GGCGGGAGGCAGGaccaaacCGGTCCGCAGTC-3’), and pmEts-b (5’-GGCGGGAGGCAGGACTaaagaCGGTCCGCAGTC-3’) were annealed (70°C, 10 min) in 100 µl of 25 mM Tris-HCl, pH 8.0, 0.5 mM MgCl₂, and 25 mM NaCl and then cooled to room temperature. An aliquot (10 pmol) of the double-stranded oligonucleotides was 5’-end-labeled with [³²P]ATP (Amersham Pharmacia Biotech, Piscataway, NJ) and T4 polynucleotide kinase and purified on a Sephadex G-25 column (Amersham Pharmacia Biotech). A DNA protein-binding reaction was performed for 30 min at room temperature in 40 µl of 1 x binding buffer (40 mM Tris-HCl, pH 7.9, 4 mM MgCl₂, 2 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol, 200 µg/ml bovine serum albumin, 20% glycerol, and 0.2% Nonidet P-40) containing 1 µg of poly(dl-dC) (Amersham Pharmacia Biotech), 1 µl of the radiolabeled probe (50,000-80,000
cpm), and nuclear extracts of COS-7 cells. In some cases, unlabeled double-stranded pEts (100-fold molar excess), anti-Ets-1 or anti-Ets-1/Ets-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-V5 antibody (Invitrogen) was included in the incubation mixture. The labeled probe was separated from DNA-protein complexes by electrophoresis on 6% nondenaturing polyacrylamide gels in Tris borate/EDTA buffer (44.5 mM Tris-HCl, pH 8.3, 44.5 mM boric acid, and 1 mM EDTA) at 4°C until the xylene cyanol dye reached 5 cm from the bottom of the gel. Autoradiography was performed by exposure of the gel to an imaging plate for 15-30 min, and images were analyzed by Fuji BAS-2000 (Fiji Photo Film Co., Ltd. (Tokyo, Japan)). The intensity of the gel-shift band was calculated using the Quantity One software (PDI, Huntington Station, NY).

**SDS-PAGE and Immunoblot Analysis** - The proteins were separated by SDS-PAGE according to the method of Laemmli (38) with 10% (w/v) gels, and transferred to a nitrocellulose membrane (Hybond-C, Amersham) with a semi-dry electroblotter (Sartorius, Goetingen, Germany). The membrane was treated with 5% (w/v) dried skim milk in 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl at 4°C overnight, washed, and then treated with anti-Ets-1/Ets-2, anti-Ets-1 or Actin (Santa Cruz Biotechnology, Inc.) antiserum diluted 1 to 200 for 2 h, followed by washing with 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl. Immunoreactive proteins were visualized by treatment for 30 min with protein A-peroxidase complex (Zymed Laboratories, San Francisco, CA) diluted 1 to 2,500 and the peroxidase immunostaining kit (Wako, Osaka, Japan).

**RT-PCR** - COS-7 cells (2.5 x 10⁵) were transfected with pcDNA (1.25 μg), pcEts-1 (1.25 μg), pcTEF-4 (1.25 μg), or pcEts-1 and pcTEF-4 (1.25 μg each) in 60-mm plates as described above. pcDNA was used so that the plasmid content in each experiment was equal. After cells had be cultured for 24 h, total mRNA was obtained with RNA extraction kits (Qiagen, Valencia, CA) according to the manufacturer's
instructions. One µg of total RNA was reverse-transcribed at 50°C for 30 min, then subjected to 25 cycles of amplification (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) using the one-step RT-PCR kit (Qiagen). The primers used for CT were 5'-ATGCACAGTGTCAGCCAA-3' (sense) and 5'-GGGCTTACTAAAGTCAACTCAA-3' (antisense), and they produce an ~200-bp CT fragment. Primers for glycero-3-phosphate dehydrogenase (G3PDH) were, 5'-TCCACCACCTGTTGCTGTA-3' (sense) and 5'-ACCACAGTCCATGCCATCAC-3' (antisense). The intensities of the CT bands were normalized to those of the G3PDH bands using the Quantity One software.

Statistical Analysis- All values are expressed as means ± S.D. Group means were compared by Student’s t test or Cochran-Cox test after analysis of variance to determine the significance of difference between the individual means. Statistical significance was assumed at p < 0.05.

RESULTS

Deletion and Mutation Analysis of Chimeric CT Promoter-Luciferase Reporters and Their Activation by TEF-4 in COS-7 Cells- To examine the promoter region for CT basal transcription, we prepared various CT promoter deletion constructs linked to the luciferase reporter and transfected into COS-7 cells. The expression of luciferase activity was determined by dual-luciferase assays and normalized for transfection efficiency after co-transfection with pRL-CMV Renilla vector. The Renilla luciferase activity of the CMV promoter-driven controls was constant and was not affected by TEF-4 throughout the experiments. As shown in Fig. 2A, when cells were transfected with CT promoter-luciferase constructs, luciferase activity was increased dependent on the length of the promoter region. The deletion analysis clearly demonstrated that constructs D3 (-52/+38) or shorter had minimal luciferase activity compared with D1.5 (-71/+38). These results suggested that important
positive regulatory regions for basal CTα transcription in COS-7 cells resided between positions -52 and -71.

To substantiate this proposal we made mutated constructs of LUC.C7 and LUC.D1.5 at each putative transcription factor binding site shown in Fig. 1. After transfection, the luciferase activities of the mutated LUC.C7 and LUC.D1.5 constructs were assayed (Fig. 2B and 2C, respectively). When the Sp1 site at -139 was mutated, the luciferase activity of the LUC.mSp1(1)/C7 was decreased significantly, but only 25% compared with LUC.C7. When the Sp1 site at -67 was mutated, the luciferase activities of LUC.mSp1(2)/C7 did not decrease compared to LUC.C7. The luciferase activity of the NF-κB site mutant (LUC.mNF-κB/C7) decreased by 25% compared to LUC.C7 (Fig. 2B). Mutation in the CREBP binding site at +20 slightly decreased the luciferase activity (Fig. 2B). However, the luciferase activities of mutated constructs in the putative Ets binding site (EBS), LUC.mEts-a/C7 and LUC.mEts-b/C7, were decreased by ~80% compared with LUC.C7 activity as shown in Fig. 2B. The expression of the shorter constructs, LUC.mEts-a/D1.5 and LUC.mEts-b/D1.5, were very low, only ~5% compared with LUC.D1.5 (Fig. 2C). These results strongly suggest that the promoter region between -53 and -47 is an important site for basal CTα transcription. We reported TEF-4 enhanced the luciferase activity of LUC.C7 and LUC.D1.5 about twice (29 and Fig. 2). When the mutations in EBS were created, co-transfection with TEF-4 cDNA also stimulated the activity of LUC.mEts-a/C7 or D1.5 and LUC.mEts-b/C7 or D1.5 about 2-fold. However, the TEF-4 enhanced luciferase activities with LUC.mEts-a/C7 or D1.5 and LUC.mEts-b/C7 or D1.5 were much smaller than LUC.C7 or LUC.C1.5. Thus, mutation of the Ets binding of the CTα promoter decreased luciferase activity to a low level (Fig. 2A, B, and C).

**DNA Binding Properties of the Nuclear Extracts of COS-7 Cells**- To test whether or not there is a protein in the nuclear extracts from COS-7 cells that binds to the
putative EBS (-49/-47), we prepared probes with or without a mutation in the EBS. As shown in Fig. 3A, lanes 2 and 3, DNA-protein complexes with the pEts (-65/-36) probe were clearly identified. When the EBS in pEts was mutated from GACTTC to ACCAAA (-53/-48) (pmEts-a) (Fig. 3A, lanes 7 and 8) or from TCC to AAA (-49/-47) (pmEts-b) (Fig. 3A, lanes 10 and 11), the bands were attenuated or disappeared. These results strongly suggested that there was a protein(s) in the extracts of COS-7 cells that bound to EBS in the pEts probe.

After transfection with pcTEF-4, the band intensities of the specific DNA-protein complexes were significantly increased (Fig. 3A, lane 2 and 3, and Fig. 3B). This result indicated that TEF-4 enhanced the binding of pEts probe and protein complexes to EBS.

Identification of Ets-1 as the Protein that Binds to the pEts Probe- To identify the protein(s) binding to pEts, we used an antibody, anti-Ets-1/Ets-2, with specificity for the DNA binding site in the C-terminus of Ets family proteins. Immunoblot analysis indicated the existence of a 54-kDa protein that cross-reacted with anti-Ets-1/Ets-2 antibody in the nuclear extracts from COS-7 cells (Fig. 4A, upper panel). When various amounts of the anti-Ets-1/Ets-2 antibody were added to incubations of nuclear extracts with the pEts probe, the intensity of the immunoreactive band decreased (Fig. 4B, left). This result indicated that the bands revealed by the gel-shift analysis resulted from a complex of Ets-1 or Ets-2 with the pEts probe. To determine which member of the Ets family was binding to pEts, we used an Ets-1 specific antibody. Immunoblot analysis showed a 54-kDa protein that cross-reacted with anti-Ets-1 antibody in the nuclear extracts from COS-7 cells (Fig. 4A, middle panel). When we used the anti-Ets-1 antibody with the probe in gel shift analyses, the decrease in the intensity of the band was similar to anti-Ets-1/-2 (Fig. 4B, right). Thus, it appears that Ets-1 is the protein that binds to the EBS of pEts.

When COS-7 cells were stimulated with TEF-4, the basal luciferase activities of
LUC.C7 and LUC.D1.5 were increased (Fig. 2) and the binding of endogenous Ets-1 to pEts was significantly enhanced (Fig. 3, lane 3). However, immunoblot analysis showed that the amount of Ets-1, or actin as the internal standard, was not changed by TEF-4 stimulation (Fig. 4A). Thus, TEF-4 might modify the binding of Ets-1 to the promoter, and enhance Ets-1-stimulated CT\(^a\) transcription.

**Over-expression of Ets-1 Increases the Binding of Ets to the CT\(^a\) promoter** - In another approach to confirm that Ets does indeed bind to the CT\(^a\) promoter, we transfected COS-7 cells with a vector that contained the cDNA encoding Ets-1 (pcEts-1) or the vector alone (pcDNA). Nuclear extracts were prepared after 48 h and incubated with labeled pEts probe (Fig. 5A). There was increased binding of a nuclear protein to the probe in extracts from cells transfected with pcEts-1 (lane 3) compared to pcDNA (lane 2). Evidence that this nuclear protein was Ets was provided by incubation of the nuclear extract with antibody to Ets-1 (anti-V5) and a super shift was observed (Fig. 5A, lane 5).

In complementary experiments, pcEts-1 or pcDNA was co-transfected with CT\(^a\) promoter-luciferase constructs LUC.C7 (-1268/+38), LUC.D2 (-130/+38) or LUC.D1.5 (-71/+38). The expression of luciferase activities was normalized to protein concentration. The vertical axis in Fig. 5B represents the fold-increase in luciferase activity after 48 h transfection relative to that obtained for the minimal promoter construct, LUC.D1.5 with pcDNA. The results in Fig. 5B show transfection with a cDNA encoding Ets-1 caused an increase in luciferase activity relative to the vector control with the longest promoter-luciferase construct (LUC.C7) exhibiting the highest fold-increase.

TEF-4 is an activating nuclear factor that binds at the –97/-89 region of the CT\(^a\) promoter (29). To test whether co-expression of TEF-4 with Ets-1 would enhance luciferase expression, we performed co-transfection experiments with plasmids that expressed these nuclear proteins and either LUC.C7 (-1268/+38) which
contains both TEF-4 and Ets-1 binding sites, or LUC.D1.5 (-71/+38), that lacks the TEF-4 binding site. It is evident from Fig. 5C that transfection with pcTEF-4 and pcEts-1 slightly stimulates the expression of luciferase with the LUC.D1.5 promoter, whereas there was more than a doubling of luciferase activity when pcTEF-4 and pcEts-1 were co-expressed with LUC.C7. Thus, the TEF-4 binding element in the CTβ promoter appears to be important in the activation by Ets-1 and TEF-4.

Expression of Ets-1 and TEF-4 Enhance the Expression of the mRNA for CTβ in COS-7 Cells- We also determined if the expression of the endogenous CTβ mRNA would be increased in cells transfected with pcEts-1, pcTEF-4, or both, compared to pcDNA. The results in Fig. 6 show an ~1.5-fold increase in the mRNA for CTβ when either pcEts-1 or pcTEF-4 was transfected into COS-7 cells, and more than a doubling of CTβ mRNA when pcEts-1 and pcTEF-4 were co-transfected.

DISCUSSION

The results of this study strongly support the proposal that Ets-1 is an important transcription factor that stimulates basal CTβ transcription. The stimulatory activity of Ets-1 is enhanced by the action of TEF-4.

Ets was originally identified as one of three transforming genes in avian erythroblastosis virus, E26, 20 years ago (36). The transforming gene consists of three elements, the myb domain, the ets domain and gag domain (36, 39). Ghysdael et al. (40) identified the c-ets proto-oncogene product as a 54-kDa cytoplasmic protein. Watson et al. (41, 42) showed that the human Ets-1 gene encodes a protein with 441 amino acids. Ets-2 cDNA encodes a closely related protein with 469 amino acids (42). Ets-1 and Ets-2 have three domains: the C-terminal conserved DNA binding domain, a N-terminal domain referred to as the “pointed domain” and a trans-activation domain between these two structures (43, 44). The Ets DNA binding domain has a winged helix-turn-helix (45). Recently, the
Ets-1 pointed domain was found to be phosphorylated with mitogen-activated protein kinase (46), and was reported to contain an ERK2 docking site (47). Currently, at least 18 Ets domain-containing genes have been identified in the human genome. EBS has also been identified in the promoter/enhancer regions of more than 200 viral and cellular genes (44). Thus, the Ets family of proteins and the EBS are implicated in the control of the expression of genes involved in cellular proliferation, development, hematopoiesis and apoptosis (44).

Ets-1 expression increases in murine embryos after implantation and during organogenesis. In later fetal stages, its expression is predominant in lymphoid tissues and brain (48). Disruption of Ets-1 expression can block the proliferative capacity and the activation of T cells (49, 50). Ets proteins have also been implicated in several malignant disorders. The specific chromosomal translocation of Ets-1 genes was reported in human leukemias (51), and several Ets genes are located at the translocation breakpoints of several patients’ leukemias and solid tumors (52). In addition, Ets factors are over-expressed in prostate cancer (53) and breast cancer (54). Vascular endothelial growth factor secreted by human glioma cells induces Ets-1 expression in adjacent endothelial cells and promotes angiogenesis (55). However, the genes that are important targets of over-expressed Ets factors in cancer development have not been identified.

In the present study, we demonstrated that Ets-1 binds to its GGAA consensus site (-47/-50) in the CTα promoter and enhances the transcription of CTα luciferase reporters in COS-7 cells. The expression of CTα is positively associated with preparation for cell division (24), in colony stimulating factor 1-stimulated macrophages (19), after partial hepatectomy (20, 21), and during the perinatal period (22). Moreover, CTα expression is enhanced in tumorigenic cells (56-58). Ets-1 may be expressed in cells and tissues in proliferative situations, then enhance the induction of CTα mRNA in preparation for cell growth.
Several factors, such as NF-κB (59), core binding factor (60), and Sp1 (61) were reported to form complexes with Ets-1 and act as enhancers or modulators of DNA binding and trans-activation by Ets-1 (62). NF-κB, Sp1, Sp2, and Sp3 binding sites are found adjacent to the Ets-1 binding site in *Ctpct* (27, 28). Mutations in either the NF-κB (-58) or Sp1 (-139) binding sites in *Ctpct* slightly, but significantly, decreased the luciferase activity of the CTα promoter reporter in COS-7 cells as shown in this study. NF-κB is a ubiquitous transcription factor involved in immune, inflammatory and stress responses (63). Adjacent or overlapping binding sites for Ets and NF-κB are present in many inducible lymphoid genes, including the IL2-receptor (64), IL3 (65), GM-CSF (66), and IL-12 (67). Thus, the combination of Ets and NF-κB may play an important role in CTα transcription. More recently, Ets-1 and Sp1 were reported to cooperatively enhance the transcription of several genes (61, 68-70). In addition, we reported the importance of Sp1 and Sp3 binding for the regulation of the CTα gene in several cell lines (27, 28), and showed more recently that Ha-Ras stimulated CTα gene transcription through Sp3 phosphorylation by p42/44MAPK (71).

Ets-1 is also a nuclear target of the Ras signaling system and phosphorylation of the pointed domain of Ets-1 is important for Ras-mediated activation of Ets-1 (47, 72). However, no evidence for enhanced binding of Ets was observed in the Ras-expressing C3H10T1/2 cells (71).

We recently reported that the binding site of TEF-4 in *Ctpct* is -97/-89 (29). However, TEF-4 enhanced the basal luciferase activity of CTα promoter-reporter constructs shorter than -71 that lacked the TEF-4 binding site. As reported here, gel-shift analysis clearly revealed that TEF-4 enhanced the binding of Ets-1 to the CTα promoter. TEF-4 was a more effective activator of Ets-1-enhanced luciferase activation with a construct that contained the TEF-4 binding site. These results suggest that TEF-4, while binding to its own element, might have some interaction with Ets-1 so as to enhance the binding ability of Ets-1 to the promoter and thereby
stimulate CT[] transcription.

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REFERENCES


FOOTNOTES

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§ The abbreviations used are: PC, phosphatidylcholine; CREBP, cAMP responsive element binding protein; CTₐ, CTP:phosphocholine cytidylyltransferase gene; Ctpct, CTP:phosphocholine cytidylyltransferase gene; EBS, Ets binding site; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; TEF-4, transcriptional enhancer factor-4.
**Figure Legends**

**FIG. 1.** **Partial structure and putative regulatory elements in the murine Ctpct promoter.** The 5'-flanking region shown is 250 bp (-212/+38), and indicated are the consensus elements for putative binding sites of nuclear factors defined by searching the TRANSFAC transcription factor database; Sp1, Transcriptional enhancer factor-4 (TEF-4), NF-κB, Ets, and cAMP response element binding protein (CREBP), are underlined. Numbers show the positions of nucleotides in relationship to the first transcription initiation site +1. The positions for the starting sites of the deletion mutants, LUC.C8(-201/+38), LUC.D2 (-130/+38), LUC.D1 (-90/+38), LUC.D1.5 (-71/+38), LUC.D3 (-52/+38), LUC.D3.25 (-43/+38), LUC.D3.5 (-31/+38), LUC.D4 (-10/+38), and LUC.D5 (+10/+38) are indicated. Negative signs (-) following Sp1(1) and Ets indicate the binding consensus site is on the opposite strand of DNA.

**FIG. 2.** **Deletion and mutation analysis of murine Ctpct promoter-luciferase reporter constructs and the effect of TEF-4 on their expression.** A, truncated CT[] promoters of LUC.C7 (-1268/+38) and LUC.C8, LUC.D2, LUC.D1, LUC.D1.5, LUC.D3, LUC.D3.25, LUC.D3.5, LUC.D4, or LUC.D5 (shown in Fig. 1) were cloned into the luciferase reporter vector pGL3-basic. Luciferase plasmids (0.5 µg), pcDNA (0.5 µg), and pRL-CMV (0.001 µg) were transfected into COS-7 cells. Reporter activities were measured 48 h after transfection and normalized for transfection efficiency as described under “Experimental Procedures”. B, LUC.C7 and the mutated CT[] promoters (LUC.mSp1(1)/C7, LUC.mSp1(2)/C7, LUC.mNF-κB/C7, LUC.mEts-a/C7, LUC.mEts-b/C7, or LUC.mCREB/C7) (0.5 µg), and pRL-CMV (0.001 µg) were transfected into COS-7 cells with pcDNA (0.5 µg) (white bars) or pcTEF-4 (0.5 µg) (black bars). TEF-4 significantly enhanced the luciferase activity of
each luciferase construct compared with vector control (p < 0.01). C, LUC.D1.5 or
the mutated CT[ promotors (LUC.mEts-a/D1.5, LUC.mEts-b/D1.5, LUC.mCREB/D1.5) (0.5 µg), and pRL-CMV (0.001 µg) were transfected into COS-7
cells with pcDNA (0.5 µg) (white bars) or pcTEF-4 (0.5 µg) (black bars). TEF-4
significantly enhanced the luciferase activity of each luciferase construct compared
with vector control (p < 0.01). Values are means ± S.D. from three different dishes.
Each experiment was repeated three times with similar results.

FIG. 3. Gel-shift analysis of nuclear extracts of COS-7 cells with the pEts or
mutated pEts (pmEts-a or pmEts-b) promoter element. A, the labeled pEts probe
(lanes 1-5), pmEts-a (lanes 6-8), or pmEts-b (lanes 9-11) was incubated with 3.5 µg
of nuclear extract from COS-7 cells transfected with control vector (lanes 2, 4, 7, and
10) or pcTEF-4 (lanes 3, 5, 8, and 11) for 48 h, and separated on 6% nondenaturing
polyacrylamide gels. One-hundred-fold molar excess of unlabeled pEts probe (lane
4 and 5) was used for competition with labeled pEts probe. The arrow indicates the
position of specific DNA-protein complexes. Bovine serum albumin was added to
equalize the protein content in each lane. Each experiment was repeated twice with
similar results. B, COS-7 cells were transfected with pcDNA (0.5 µg) or pcTEF-4 (0.5
µg). Nuclear extracts were obtained 48 h after transfection, and 3.5 µg of nuclear
extracts were separated on 6% nondenaturing polyacrylamide gels. The band
intensities of the specific DNA-protein complexes as shown in A (lane 2 and 3)
were quantified as described under “Experimental Procedures”. * represents p <
0.01 compared with the vector control. Values are means ± S.D. from three
independent dishes. Each experiment was repeated twice with similar results.

FIG. 4. Immunoblot and gel-shift analyses of nuclear extracts of COS-7
cells with anti-Ets antibodies. A, COS-7 cells were transfected with pcDNA (0.5
μg) or pcTEF-4 (0.5 μg). Nuclear extracts were obtained 48 h after transfection, and 21 μg of nuclear extracts were separated by SDS-PAGE and then subjected to immunoblot analysis using anti-Ets-1/Ets-2 antibody (upper panel), anti-Ets-1 antibody (middle panel), or anti-actin antibody (lower panel) as described under “Experimental Procedures.” The large arrows indicate the position of molecular mass markers. B, the labeled pEts probe was incubated with 3.5 μg of nuclear extract from COS-7 cells and separated on 6% nondenaturing polyacrylamide gels. Nuclear extracts were incubated without (lane 1) or with 4 (lane 2), 8 (lane 3), 16 (lane 4), or 32 (lane 5) μg of anti-Ets-1/-2 antibody, or with 4 (lane 6), 8 (lane 7), 16 (lane 8), or 32 (lane 9) μg of anti-Ets-1 antibody. Bovine serum albumin was used to equalize the protein content in each lane. The arrow indicates the position of specific DNA-protein complex.

**FIG. 5.** Over-expression of Ets-1 enhances the binding of nuclear extracts of COS-7 cells to the pEts promoter elements and stimulates murine CTα promoter-luciferase reporter constructs. A, COS-7 cells were transfected with pcDNA (0.5 μg) or pcEts-1 (0.5 μg). The labeled pEts probe was incubated with 3.5 μg of nuclear extracts from COS-7 cells, and separated on 6% non-denaturing polyacrylamide gels. Nuclear extracts were incubated without or with 1 μg anti-V5 antibody. Bovine serum albumin was added to equalize the protein content in each lane. The lower arrow indicates the position of specific DNA-protein complex, and the upper arrow indicates the position of specific DNA-protein-antibody complex. B, LUC.C7, LUC.D2, or LUC.D1.5 (0.5 μg) were transfected into COS-7 cells with pcDNA (0.5 μg) (white bars), or pcEts-1 (0.5 μg) (oblique cross striped bars). The vertical axis represents the fold-increase in luciferase activity after 48 h transfection relative to that obtained for the minimal promoter construct, LUC.D1.5 with pcDNA.
Ets-1 significantly enhanced the activity of each luciferase construct compared with vector control ($p<0.01$). Values are means ± S.D. from three independent dishes. Each experiment was repeated three times with similar results. C, LUC.C7 or LUC.D1.5 (0.5 µg) were transfected into COS-7 cells with pcDNA (0.5 µg) (white bars), pcTEF-4 (0.5 µg) (black bars), pcEts-1 (0.5 µg) (oblique cross striped bars), or pcEts-1 and pcTEF-4 (0.5 µg) (crosswise striped bars). The vertical axis represents the fold increase in luciferase activity after 48 h transfection relative to that obtained for the minimal promoter construct, LUC.D1.5 with pcDNA. pcTEF-4, pcEts-1, or pcTEF-4 and pcEts-1 significantly enhanced the luciferase activity of each luciferase construct compared with vector control ($p<0.01$). Values are means ± S.D. from three independent dishes. Each experiment was repeated three times with similar results.

**FIG. 6. Ets-1 and TEF-4 increase CTα mRNA abundance.** A, COS-7 cells were transfected with pcDNA (1.25 µg), pcEts-1 (1.25 µg), pcTEF-4 (1.25 µg), or pcEts-1 and pcTEF-4 (1.25 µg each). Total mRNA was obtained 24 h after transfection, and 1 µg of total RNA was used for RT-PCR analysis of mRNA encoding CTα and G3PDH. B, the band intensities in A were quantified as described under “Experimental Procedures”. The values for G3PDH mRNA were used to normalize the band density of CTα mRNA. The over-expression of Ets-1 and/or TEF-4 significantly enhanced CTα transcription ($p<0.05$). Values are means ± S.D. from three independent dishes. Each experiment was repeated twice with similar results.
Fig. 1

-212 ACACATCCGGAATTCCGAGGCCCGACCCCGCGCTGCTTCCAGCGTTC
  C8(-201)

SREBP
(-156/-147)

Sp1(1)(-)
(-139/-136)

-162 GGCTCAAGTCACCCACGCGCCCGGCCCTCTGGAAGCGGAACCTACTCTGT
  D2(-130)

TEF-4
(-97/-89)

Sp1(2)
(-67/-62)

-112 CAGGTTGTGGTTTTCAGGAATGCGGAGGTGGCATTGACAAGAGGGCCGGGC
  D1(-90)

NF-kB
(-58/-54)

Ets(-)
(-49/-47)

-62 GGGAGGCGGGACGCCGGTCCCGCAGTCCGGGTCAAGATGTTTCCCGGGCGTC
  D3(-52)  D3.25(-43)  D3.5(-31)

CREB
(+20/+23)

-12 TCCCCCGCAACCCATTGGACTTGCTAGTCCGGATGCAGGCAGGCGGGCGGCCGGAAGG
  D4(-10)  +1  D5(+10)  +35
Luciferase Activity
(Photinus pyralis/Renilla reniformis)

Fig. 2
Fig. 4

**A**

- Anti-Ets-1/-2
- kDa:
  - 121
  - 81
  - 51.2
  - 33.6
- Anti-Ets-1
- Anti-Actin
- pcDNA  pcTEF-4

**B**

- Anti-Ets-1/-2
- Anti-Ets-1

Probe:  pEts
Fig. 5

A

pcDNA  pcEts-1

Anti-V5

+B

D1.5

C7

C7

D1.5

B

D2

pcDNA

pcEts-1

pcDNA

pcEts-1

pcDNA

pcEts-1

C

pcDNA

pcEts-1+pcTEF-4

pcEts-1+pcTEF-4

pcEts-1+pcTEF-4

pcEts-1+pcTEF-4

probe: pEts

(-fold increase)

0

5

10

15

20

25

30

D1.5
Fig. 6

A

CT

G3PDH

B

pcDNA  pcTEF-4  pcEts-1  pcEts-1+pcTEF-4

pcDNA  pcTEF-4  pcEts-1  pcEts-1+pcTEF-4
Identification of Ets-1 as an important transcriptional activator of CTP:Phosphocholine cytidylyltransferase a in COS-7 cells and Co-activation with transcriptional enhancer factor-4
Hiroyuki Sugimoto, Sayaka Sugimoto, Tatei Kazuaki, Hideru Obinata, Marica Bakovic, Takashi Izumi and Dennis E. Vance

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