Sp1 Is a Co-activator with Ets-1, and Net Is an Important Repressor of the Transcription of CTP:Phosphocholine Cytidyltransferase α*

Hirohito Sugimoto†‡, Koichi Okamura†, Sayaka Sugimoto§, Motoyasu Satou†, Tomoyasu Hattori†, Dennis E. Vance†‡, and Takashi Izumi¶§

From the †Department of Molecular Biochemistry, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan, the §Department of Biochemistry, Dokkyo University School of Medicine, Mibu 321-0293, Japan, and the ¶Department of Biochemistry and Canadian Institutes of Health Research Group on Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta T6G 252, Canada

Phosphatidylcholine biosynthesis via the CDP-choline pathway is primarily regulated by CTP:phosphocholine cytidylyltransferase (CT) encoded by the Pcyt1a and Pcyt1b genes. Previously, we identified an Ets-1-binding site located at −49/−47 in the promoter of Pcyt1a as an important transcriptional element involved in basal CTα transcription (Sugimoto, H., Sugimoto, S., Tatei, K., Obinata, H., Bakovic, M., Izumi, T., and Vance, D. E. (2003) J. Biol. Chem. 278, 19716–19722). In this study, we determined whether or not there were other important elements and binding proteins for basal CTα transcription in the Pcyt1a promoter, and if other Ets family proteins bind to the Ets-1-binding site. The results indicate the formation of a ternary complex with Ets-1 binding at −49/−47 and Sp1 binding at −58/−54 of the Pcyt1a promoter that is important for activating CTα transcription. When nuclear extracts of COS-7 cells expressing various Ets family repressors were incubated with DNA probes, binding of Net to the probes was observed. Net dose-dependently decreased the promoter-luciferase activity by 98%, even when co-expressed with Ets-1. RNA interference targeting Net caused an increase of endogenous CTα mRNA. After synchronizing the cell cycle in NIH3T3 cells, CTα mRNA increased at the S-M phase corresponding to an increase of Ets-1 mRNA and a decrease of Net mRNA. These results indicated that Net is an important endogenous repressor for CTα transcription.

Phosphatidylcholine (PC) is the major membrane phospholipid in mammalian cells and tissues and is produced in all nucleated cells via the CDP-choline pathway (1–4). An important rate-limiting and regulated enzyme in this pathway is CTP:phosphocholine cytidylyltransferase (CT), and cDNAs of four isoforms for CT have been reported (5–8). The most active and ubiquitous form of CT in cells and tissues is CTα, which was purified (9, 10) and cloned (5). cDNAs for three other isoenzymes, CTβ1, CTβ2, and CTβ3, were recently cloned and are encoded by a second gene (6–8). Regulation of CTα activity has been extensively studied. The helical domain for the binding of specific lipids is important for translocation between the membrane and the cytosol (11), and a highly phosphorylated domain at the C terminus can modulate its enzyme activity (12). In addition to post-translational regulating mechanisms, CTα mRNA expression is strictly regulated. Its expression is increased in colony-stimulating factor-1-stimulated macrophages (13) during liver development (14), after partial hepatectomy of rat liver (15, 16), and during S phase of the cell cycle (17).

The murine CTα gene (Pcyt1a) was isolated, and its exon/intron organization resembles the functional domains of the enzyme (18). The gene is transcribed from two transcriptional start sites and lacks a TATA box but contains a GC-rich region that is characteristic of many other TATA-less promoters (19). The binding sites for Sp1, Sp3 (19, 20), transcriptional enhancer factor-4 (TEF-4) (21), Ets-1 (22), and sterol-response element-binding protein (23, 24) were reported in the 200-bp proximal promoter. Three Sp1-binding core elements, −139/−136, −67/−62, and −19/−14, have been identified, and they are involved in basal, activator, and suppressor activities. Sp1, Sp2, and Sp3 can competitively bind to these elements and regulate the expression of the CTα gene (20). When C3H10T1/2 fibroblasts were stably transfected with a cDNA encoding Ras, there was increased binding of Sp3 to the −67/−62 region. Thus, Sp3 is an important factor for CTα expression in these fibroblasts (25). Banchio et al. (26) recently reported that CTα expression during the S phase of the cell cycle is mediated by Sp1 phosphorylation by cyclin-dependent kinase 2 and binding of Sp1 to the −67/−62 region.

Bakovic et al. (19) reported that the murine CTα gene promoter is positively regulated by an enhancer element (Eb) located between −103 and −83 bp, and there was a protein bound to Eb in nuclear extracts of HeLa cells. We identified TEF-4 as that binding protein (21). It was interesting that TEF-4 enhanced the basal transcription of CTα in COS-7 cells by interactions with elements shorter than −71 that do not contain TEF-4 binding site. When we determined the main transcriptional region involved in basal CTα transcription, Ets-binding core element −49/−47 (EBS) was identified as the binding site of Ets-1, and its stimulating activity was enhanced by TEF-4 (22). In this study, we determined whether or not there were other important elements and binding proteins for basal CTα transcription, and whether other Ets family proteins bind to the EBS. We identified a newly recognized important site...
for basal CTA transcription located at −58/−54, which was clarified as an additional binding site for Sp1. We demonstrate that Sp1 binding at −58/−54 interacts with Ets-1 binding at the EBS and promotes CTA transcription. We also identify Net, an Ets-related protein, as a potent inhibitor of CTA transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—The luciferase vector pGL3-basic that contains the cDNA for *Photinus pyralis* luciferase, the control pRL-CMV or pRL-TK vector that contains the cDNA for *Renilla reniformis* luciferase, and the dual-luciferase reporter assay system were obtained from Promega (Madison, WI). FuGENE 6™ transfection reagents, Dulbecco’s modified Eagle’s medium (DMEM) (high glucose), and fetal bovine serum (FBS) were from Roche Applied Science, Sigma, and Invitrogen, respectively. COS-7 and NIH3T3 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

**Construction of Ets-1, Elk-1, Net, Mutated Net, ERF, Fli-1, Tel, and Net Fused with GFP Expression Vectors**—The mouse full-length cDNA coding murine Ets-1 was obtained as reported before (22). The mouse full-length cDNAs coding murine Elk-1, Net, ERF, Fli-1, and Tel were obtained through PCR of a reverse-transcribed product of mRNA from NIH3T3 cells using Superscript II (Invitrogen). PCR was performed with the complementary primers, 5′-CTAGTATGACCCCATCTCTG-TGACGC-3′ and 5′-TGGCTTCTGGGGCCCTGGGGAGAGC-3′ for Elk-1; 5′-CTGGTGTATGGAGATGTCAATCACGC-3′ and 5′-GGATTTCTGAGCTGGGGACAGC-3′ for Net; 5′-CCCCAGCATGAGACCCCCGGCGGACAC-3′ and 5′-GAATCTCGCTGTTCCAGGGAGATGGT-3′ for ERF; 5′-GGCCAAATGGACGGGACTATAAAG-3′ and 5′-GTAGTATGCTCCTAATTGTAAGGAC-3′ for Fli-1; and 5′-GTGAGATGTGAGCTGCTC-3′ and 5′-GGAATCTCGGTGCTCCAGA-3′ for Tel with Platinum II polymerase. The sequences were confirmed.

**Preparation of Deleted and Mutated CTα Promoter—Netm1** (for Netm1), in which two lysine residues (Lys-49 and Lys-51 in the nuclear localization signal (NLS) of Net) were replaced with asparagine residues, was created from pcNet by PCR using QuikChange™ site-directed mutagenesis kits (Stratagene, La Jolla, CA) with the complementary primers 5′-GGCGGGAaacaaGACTTCCGGTCC-3′ and 5′-GGCGGGAaacaaGACTTCCGGTCC-3′ for pcNet and pcNetm1, respectively. pcNetm1 was subcloned into pEGFP-C2 (Clontech) to fuse the recombinant proteins at the C terminus of GFP, and the resultant expression vectors were named pEGFP-Net (for the expression of GFP-Net) and pEGFP-Netm1 (for GFP-Netm1), respectively.

**Preparation of Deleted and Mutated CTα Promoter—Luciferase reporters**—Various 5′-deleted CTα promoter regions, LUC.C7 (−1268/+38), LUC.C8 (−201/+38), LUC.D2 (−130/+38), LUC.D1.5 (−71/+38), and LUC.D3 (−52/+38), inserted into the promoter-less luciferase vector pG53-basic (Promega), were prepared as described previously (19). To prepare mutated promoters, GGGCGG (−58/−54) was mutated to AACAA and named LUC.c7-c or LUC.md1.5-c, TCC (−49/−47) was converted to AAA and named LUC.mC7-c or LUC.md1.5-b, and both GGGCGG (−58/−54) and TCC (−49/−47) were changed to AACA and AAA, respectively, and named LUC.mC7-cb or LUC.md1.5-cb. These mutations were created from LUC.C7 or LUC.D1.5 by PCR using QuikChange™ site-directed mutagenesis kits. The mutated complementary primers were used for transformation of Escherichia coli strain JM109 cells. The sequences of all deleted and mutated constructs were confirmed.

**Tissue Culture, Transfection, and Luciferase Assays—CO5-7 or NIH3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells (2.5 × 10⁵) were plated on 60-mm plates (Discovery Labware) and grown overnight. Seven and a half μl of FuGENE 6™ was suspended in 250 μl of serum-free medium and mixed with 0.125, 0.4, or 1.25 μg of pcEts-1, pcNet, pcERF, pcFli-1, or pcTel, or pcDNA for preparation of nuclear extracts to perform gel-shift analyses, immunoblot analysis, or total mRNA for RT-PCR.**

For luciferase assays, cells (1 × 10⁵) were plated on 35-mm plates 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 CTα promoter-luciferase constructs (see above), 0.001 μg of pRL-CMV or pRL-TK Renilla vector as a transfection control, and 0.005–0.5 μg of either pcEts-1, pcSp1, pcElk-1, pcNet, pcERF, pcFli-1, pcTel, pcNetm1, or pcDNA. Transfection was initiated by dropwise addition of DNA suspension to the cell culture. Twenty four or 48 h later, cells were harvested and lysed in 200 μl of Passive lysis buffer (Promega), and 5 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 CTα promoter mutated or deletion constructs (see above) and the pRL-CMV or pRL-TK control carrying the cytomegalovirus or thymidine kinase promoter-luciferase fusion. When the effects of Sp1 on CTα promoter were examined, luciferase values were normalized against total protein concentration determined by protein assay (Bio-Rad) as the Renilla luciferase activity was affected by the transfection of pcSp1.

**Synchronization of the Cell Cycle—NIH3T3 cells (1 × 10⁵) were plated on 60-mm plates and grown overnight. Twenty four h later, DMEM with 10% FBS was changed to DMEM with 0.5% FBS. After 3 days of serum starvation, the medium was changed to DMEM with 10% FBS. To analyze the phase of the cell cycle at the indicated time after 10% FBS supplementation, cells were fixed with 70% ethanol and then stained with 50 μg/ml propidium iodide (Sigma). After cells were stained, flow cytometric analysis was performed using Epics XL (Beckman Coulter, Inc., Fullerton, CA). To analyze the cellular localization of Net, transfection was initiated by dropwise addition of pEGFP, pEGFP-Net, or pEGFP-Netm1 (0.5 μg) and FuGENE 6™ suspension to the cell culture 2 h after 10% FBS supplementation. Twelve h after transfection, GFP images were obtained with a Leica DM IBERE fluorescent microscopy (Leica microsystems, Wetzlar, Germany) with a filter set for GFP.

**Preparation of Nuclear Extracts and Electromobility Gel-shift Assays—CO5-7 cells were cultured and transfected with plasmids as described above. After 48 h, nuclear extracts from COS-7 cells were prepared according to Andrews and Faller (27) with minor modifications (19). Five hundred pmol of the opposite strands of p5′ (5′-GGCGGGGGGGGGGAGTTTCCGGTCC-3′), pmS-c (5′-GGGGGGGGGGGGGGGAAacaaGACTTCCGGTCC-3′), pmS-b (5′-GGGGGGGGGGGGGGGGAGGACTTCCGGTCC-3′), pmE-c (5′-GGGGGGGGGGGGGGGGGACTTCCGGTCC-3′), pmE-b (5′-GGGGGGGGGGGGGGGGGACTTCCGGTCC-3′), and pmE-cb (5′-GGGGGGGGGGGGGGGGGGGGGAGT-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 Biosciences) and T4 polynucleotide kinase and purified on a Sephadex G-50 column (Amersham Biosciences). A DNA protein-binding reac-
tion was performed for 30 min at room temperature in 40 μl of 1× 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(di-C) (Amer- sham Biosciences), 1 μl of the radiolabeled probe (50,000–80,000 cpm), and nuclear extracts of COS-7 cells. In some cases, unlabeled double-stranded pS (100-fold molar excess), anti-Ets-1/2 antibody, anti-Sp1 antibody, anti-NF-κB antibody (Santa Cruz Biotechnology, 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 the images were analyzed by Fuji BAS-2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

**RNAi Analysis**—dsRNAs named Random (5’-CGAUUGCUCAGAACCCGCUUACUAUGC-3’) as a control and Net-878 (5’-GCAAAAAA-CCCAAGGAUAAGAAU-3’) from 878 to 903 nucleotide sequence from ATG of cDNA were obtained. NIH3T3 cells (1 × 10⁵) were plated on 35-mm dishes, and medium was changed to 0.5 ml of Opti-MEM (Invitrogen) after 24 h. Five μl of Lipofectamine™2000 (Invitrogen) was suspended in 250 μl of Opti-MEM and then mixed with 100 pmol of dsRNA. Transfection was initiated by dropwise addition of dsRNA suspension to the cell culture to a final concentration of 50 nM in the medium. Opti-MEM containing 30% FBS (625 μl) was added to the medium 4 h later, and total RNA was obtained after 24, 48, and 72 h.

**RT-PCR**—COS-7 and NIH3T3 cells were cultured and transfected as described above. After cells were cultured for the indicated times, total RNA was obtained with RNA extraction kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. For quantification of the amounts of mRNA of CTα, Ets-1, Net, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in COS-7 or NIH3T3 cells, 0.5 μg of total RNA was reverse-transcribed at 50 °C for 30 min and then subjected to 31 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s) using one-step RT-PCR kit (Qiagen) and DNA Engine Opticon 2 system (MJ Research, MA). The primers used for CTα were 5’-ATG-CACAGCTTCCAGCAACAA-3’ for COS-7 cells or 5’-ATGCAACA-GAGTGGTCATGAAGAAG-3’ for NIH3T3 cells (sense) and 5’-GGCGGC-TACTAAAGCTAATTCAGC-3’ (antisense), and they produced an ~200 bp CTα fragment. The primers of RT-PCR analysis for Ets-1 were 5’-GCCACATGAAGGGGCGCTGATC-3’ (sense) and 5’-GTTGTTTGGAGTTCCACTGGTCG-3’ (antisense), for Net were 5’-GTACTAACAACATTCGAGCGATCC-3’ (sense) and 5’-CTTGGGTATGGGA-GAGTGGTACAC-3’ (antisense), and for G3PDH were 5’-TCCTGACACCCCTGTGTA-3’ (sense) and 5’-ACCACAGTCCTGTAC-3’ (antisense). The contents of CTα, Ets-1, or Net mRNAs were normalized to those of the G3PDH mRNA using DNA Engine Opticon 2 system.

**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**

**Gel-shift Analysis of Nuclear Extracts of COS-7 Cells with pS and pE Promoter Elements**—As we reported before (21, 22), the important promoter element for basal CTα transcription in COS-7 cells is from −71 to −43, and we identified Ets-1 binding to −53/−44 as one of the important activators. When we searched the CTα promoter region using the TRANSFAC transcription factor data base, several consensus elements for the binding sites of transcription factors were identified (22). Sp1 was suggested to bind to −62/−50 regions (Fig. 1A), and NF-κB was also suggested to bind to −55/−46. To test whether or not there were other proteins that bind to the Poyl1a promoter elements from −70 to −36 in nuclear extracts, we prepared pS (−70/−42) and pE (−65/−36) probes (Fig. 1B and C). After transfection with pcEts-1 into COS-7 cells for 48 h, nuclear extracts of COS-7 cells were incubated with pS. There were slow moving bands indicated by arrow b as shown in Fig. 1B, lanes 1 and 2. The band disappeared when Sp1 binding consensus GGCGG (−58/−54) was mutated to AAAAC as in pmS-c (Fig. 1B, lanes 9 and 10). To confirm what kind of nuclear proteins bind to pS, nuclear extracts were incubated with anti-Sp1 (Fig. 1B, lanes 5 and 6) or anti-NF-κB (lanes 7 and 8). After incubation with anti-Sp1, the band intensity was much decreased (Fig. 1B, lanes 5 and 6), and supershifts indicated by arrow a appeared in Fig. 1B, lanes 5 and 6. However, when nuclear extracts were incubated with anti-NF-κB (Fig. 1B, lanes 7 and 8), the intensity of the band did not change compared with lanes 1 and 2, respectively. These results indicated that the promoter element from −58 to −54 is an important site for Sp1 binding. When nuclear extracts were incubated with anti-Sp1, the upper slow moving band indicated in Fig. 1B, arrow b, clearly decreased its density, and weak supershifts indicated by arrow a were detected in lanes 5 and 6. Therefore, the anti-Sp1 antibody was thought to have two effects on Sp1 binding to the DNA probe: inhibition of binding and formation of a larger complex detected as a super gel-shift. When EBS core element (−49/−47) was mutated to AAA as in pmS-b, the intensity of the bands for Sp1 binding did not change in Fig. 1B, lanes 11 and 12, compared with lanes 1 and 2, respectively. Therefore, the pS element is sufficient for Sp1 binding, and EBS (−49/−47) is not necessary for direct Sp1 binding to pS.

After transfection with pcEts-1 into COS-7 cells for 48 h, nuclear extracts were prepared and incubated with the pE probe. There were slow moving bands indicated by arrow a in Fig. 1C, lanes 1 and 2, and the band intensities of the DNA-protein complexes were increased after transfection of pcEts-1. There were faster moving bands indicated by arrow b after pcEts-1 transfection in Fig. 1C, lanes 2, 4, and 6. The intensity of both upper and lower bands was clearly decreased after incubation of nuclear extracts with pE, and weak shifts indicated by arrows c and d in Fig. 1C were recognized in lanes 3 and 4, respectively. After incubation of nuclear extracts with anti-Ets-1/2, the intensities of both upper and lower bands were also decreased (Fig. 1C, lanes 5 and 6 compared with lanes 1 and 2, respectively). These results indicate that the upper and lower bands indicated by arrows a and b in Fig. 1C consist of DNA-protein complexes of Ets-1, Sp1, and pE DNA probe with or without other protein(s), respectively.

To confirm the binding elements for Ets-1 and Sp1 in pE, we prepared three mutated probes, pmE-c mutated at Sp1-binding consensus core (−58/−54) (Fig. 2B, lanes 3–10), pmE-b mutated at EBS core element (−49/−47) (Fig. 1C, lanes 7 and 8), and pmE-cb mutated at both sites (Fig. 1C, lanes 9 and 10). As shown in Fig. 1C, when nuclear extracts were incubated with pmE-b, the slow moving bands in lanes 7 and 8 indicated by arrow e were weaker than the band at the same position in lanes 1 and 2. When nuclear extracts were incubated with pmE-cb, the slow moving bands in Fig. 1C, lanes 7 and 8, completely disappeared. These results indicate that both −58/−54 and −49/−47 elements are important for Ets-1 and Sp1 binding.

**Sp1 and Ets-1 Co-operatively Bind to pE**—To confirm the above hypothesis, nuclear extracts from COS-7 cells transfected with pcEts-1 were incubated with anti-V5 antibody to recognize V5-tagged Ets-1. There was a fast moving band indicated by arrow b in lane 2, Fig. 2A.
Ets-1 and Sp1 Enhance and Net Represses CTα Transcription

The supershift in Fig. 2A, lane 4, indicated by arrow c appeared after incubation with anti-V5 antibody, and the lower band in lane 2 disappeared. When anti-Sp1 antibody was incubated with nuclear extracts, two bands indicated by arrows a and b in Fig. 2A decreased their intensities, and a shift indicated by arrow d was newly recognized (lane 6). When both anti-Sp1 and anti-V5 antibodies were incubated with nuclear extracts, the slow moving band in Fig. 2A, lane 4 Fig. 2A indicated by arrow c and the fast moving band in lane 6 indicated by arrow d decreased (lane 8). These results strongly suggest that the faster moving band indicated from arrow d (Fig. 2A, lane 6) is the complex of Ets-1 and pE DNA probe, and these results strengthen the possibility that the faster moving band indicated by arrow b (lane 2) is the ternary complex of Ets-1, Sp1, and pE DNA probe. The bands indicated by Fig. 2A, arrow a, in all lanes might be a complex of Ets-1, Sp1, pE DNA probe, and some other protein(s).

For comparison of the band positions in gel-shift analysis, we show results with both pE (lanes 1 and 2) and pmE-c (lanes 9–10) in Fig. 2B. After transfection with pcEts-1, there was a clear increased band indicated by arrow b in Fig. 2B, lane 1, as in A, lane 2. When nuclear extracts were incubated with pmE-c mutated at the Sp1-binding con-
pE was mutated as in Fig. 1C, lanes 7 and 8. However, unexpectedly, the mutation at Sp1-binding core (−58/−54) in pE did not affect the fast moving band in Fig. 2B, lane 4, indicating that with the pE probe the EBS element is important for making up the ternary complex, whereas the Sp1-binding core is not. EBS was not a critical site for direct Sp1 binding to pS that has enough length for Sp1 binding as described above. These results suggest that pE probe does not have enough length for direct Sp1 binding, but Ets-1 may support the binding of Sp1 to pE through an Sp1 and Ets-1 interaction.

Deletion and Mutation Analysis of Chimeric CTα Promoter-Luciferase Reporters and Their Activation by Ets-1—To examine the important elements for CTα transcription, we prepared various CTα promoter deletion constructs linked to the luciferase reporter, and mutated LUC.C7 (−1268) and LUC.D1.5 (−71) constructs, and transfected into COS-7 cells. When COS-7 cells were transfected with pcEts-1, the luciferase activities of LUC.C7 increased 2–3-fold according to the amounts of transfected plasmids (Fig. 3A).

When cells were transfected with CTα promoter-deletion luciferase constructs, luciferase activity was increased depending on the length of the promoter region. Ets-1 significantly enhanced the luciferase activities in the constructs longer than LUC.D1.5 (−71) (Fig. 3B). However, overexpression of Sp1 did not have a significant effect on the luciferase activities in the constructs shorter than LUC.C8 (−201), and the effects were much weaker than those of Ets-1.

When the Sp1 site at −58/−54 (LUC.mC7-c or LUC.mD1.5-c) was mutated, basal luciferase activities decreased by −50% (Fig. 3, C and D). Even after pcEts-1 transfection, the luciferase activity with LUC.mC7-c was not enhanced (Fig. 3C). Basal luciferase activities of LUC.mC7-b and LUC.mD1.5-b, mutated at −49/−47 EBS core element, were less than those of LUC.mC7-c and LUC.mD1.5-c, respectively. However, pcEts-1 transfection stimulated luciferase activities of LUC.mC7-b and LUC.mD1.5-b. Furthermore, basal and Ets-1-stimulated luciferase activities of LUC.mC7-cb and LUC.mD1.5-cb decreased by −80% compared with those of nonmutated constructs. When NIH3T3 cells were transfected with CTα promoter deleted and mutated constructs, the promoter elements from −71 to −52 and both −58/−54 and −49/−47 sites were important for CTα transcription as in COS-7 cells (Fig. 3E). Together with the results as described above, we proposed that Ets-1 binding to −49/−47 is most important for CTα transcription, and Sp1 is necessary for maintaining the effects of Ets-1. However, overexpression of Sp1 did not significantly change the luciferase activity because Sp1 may be sufficiently expressed in COS-7 cells.

DNA Binding Properties of Ets Family Repressors, Net, ERF, Fli-1, and Tel to pE Probe, and Their Effects on Chimeric CTα Promoter-Luciferase Reporters—There are numerous reports about the properties of Ets family members. Although every Ets family member has a homologous Ets DNA binding domain in their molecule, one exhibits a transcriptional activator effect, whereas another exhibits a transcriptional repressor effect on the same EBS within a promoter (28). Therefore, we were interested in whether or not other Ets family repressors bind to pE probe and alter Pcyt1α expression at the transcriptional level. We transfected COS-7 cells with a vector that contained the cDNA encoding Elk-1 (pcElk-1), Net (pcNet), ERF (pcERF), Fli-1 (pcFli-1), and Tel (pcTel) with the V5 tag or the vector alone (pcDNA). Most of the known ETS proteins (Ets-1, Ets-2, and Elk-1, etc.) have been shown to activate transcription (29, 30). However, four ETS proteins (Net, ERF, Fli-1, and Tel) were reported to act as transcriptional repressors (28, 31). When nuclear extracts from COS-7 cells transfected with pcNet, pcERF, pcFli-1, or pcTel were incubated with pE, there was obvious binding of nuclear proteins to the probe in extracts from cells transfected with
Figure 3. Deletion and mutation analysis of murine Pcyt1a promoter-luciferase reporter constructs and the effect of Ets-1 or Sp1 on their expression. A, truncated CTα promoter LUC.C7 (−1268/+38) was cloned into the luciferase reporter vector pGL3-basic. LUC.C7 (0.5 μg), pRL-CMV (0.001 μg), and several amounts of pcDNA or pcEts-1 (0.005, 0.02, 0.05, or 0.25 μg) were transfected into COS-7 cells. Reporter activities were measured 24 h after transfection, normalized for transfection efficiency by pRL-CMV, and expressed as % of vector control. B, truncated CTα promoter LUC.C7 (−1268/+38), LUC.C8 (−201/+38), LUC.D1.5 (−71/+38), and LUC.D3.1 (−52/+38) were cloned into the luciferase reporter vector pGL3-basic. pcDNA (white bar), pcEts-1 (oblique cross-striped bar) or pcSp1 (crosswise striped bar) (0.5 μg) were transfected into COS-7 cells with truncated luciferase plasmids (0.5 μg). Reporter activities were measured 48 h after transfection and normalized for transfection efficiency by protein. C, truncated CTα promoter LUC.C7 (−1268/+38) and its mutated constructs LUC.mC7-c (AACAA:+58/−54), LUC.mC7-b (AA+A:−49/−47), and LUC.mC7-db (AAA−:−58/−54 and AAA−:−49/−47) were cloned into the luciferase reporter vector pGL3-basic. Luminescence plasmids (0.5 μg), pRL-CMV (0.001 μg), and pcDNA (white bar) or pcEts-1 (oblique cross-striped bar) (0.5 μg) were transfected into COS-7 cells. Reporter activities were measured 48 h after transfection and normalized for transfection efficiency relative to pRL-CMV. D, truncated CTα promoter LUC.D1.5 (−71/+38) and its mutated constructs LUC.mD1.5-c (AACAA:+58/−54), LUC.mD1.5-b (AA+A:−49/−47), and LUC.mD1.5-db (AAA−:−58/−54 and AAA−:−49/−47) were cloned into the luciferase reporter vector pGL3-basic. Luciferase plasmids (0.5 μg), pRL-CMV (0.001 μg), and pcDNA (white bar) or pcEts-1 (oblique cross-striped bar) (0.5 μg) were transfected into COS-7 cells. Reporter activities were measured 48 h after transfection and normalized for transfection efficiency relative to pRL-CMV. E, truncated and mutated CTα promoter LUC.C7, LUC.D1, LUC.D1.5, LUC.D3, LUC.mC7-c, LUC.mC7-b, or LUC.mC7-db (0.5 μg) were transfected with pRL-CMV (0.001 μg) and pcDNA (0.5 μg) into NIH3T3 cells. Reporter activities were measured 48 h after transfection and normalized for transfection efficiency relative to pRL-CMV. Values are means ± S.D. from three independent dishes. Each experiment was repeated three times with similar results.

Figure 4. Gel-shift analysis of nuclear extracts of COS-7 cells transfected with ETS protein expression vectors, pcNet, pcERF, pcP1, or pcTel and their effects on the murine CTα promoter-luciferase reporter constructs. A, COS-7 cells were transfected with pcDNA (1.25 μg) (lanes 1 and 2), pcNet (1.25 μg) (lanes 3, 4, 11, and 12), pcERF (1.25 μg) (lanes 5 and 6), pcP1 (1.25 μg) (lanes 7 and 8), or pcTel (1.25 μg) (lanes 9 and 10). Nuclear extracts were obtained 48 h after transfection, incubated with the labeled pE (lanes 1–10) or pmE-b probes (lanes 11 and 12), and separated on 6% nondenaturing polyacrylamide gels. Nuclear extracts were incubated with 1.0 μg of anti-V5 antibody to Ets-1 (lanes 2, 4, 6, 8, 10, and 12). The arrows and star indicate the positions 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, LUC.C7 (−1268/+38) (0.5 μg) and pRL-TK (0.001 μg) were transfected into COS-7 cells with pcDNA (0.5 μg) (white bar), pcElk-1 (0.5 μg) (oblique cross-striped bar), pcNet (0.5 μg) (black bar), pcERF (0.5 μg) (horizontal striped bar), pcP1-1 (0.5 μg) (crosswise striped bar), or pcTel (0.5 μg) (vertical striped bar). Reporter activities were measured 48 h after transfection and normalized for transfection efficiency relative to pRL-TK. C, LUC.C7 (−1268/+38) (0.5 μg) and pRL-TK (0.001 μg) were transfected into COS-7 cells with several amounts of pcDNA (white bar) or pcNet (black bar) (0.005, 0.01, 0.02, 0.05, or 0.5 μg). Reporter activities were measured 24 h after transfection, normalized for transfection efficiency relative to pRL-TK, and expressed as % of vector control.

pcNet (Fig. 4A, lane 3) and pcP1-1 (Fig. 4A, lane 7) compared with pcDNA (Fig. 4A, lane 1). Evidence that this nuclear protein was Net or Fli-1 was provided by incubation of the nuclear extract with anti-V5 antibody to V5-tagged Net or Fli-1 with the supershifted bands indicated by the upper arrow in Fig. 4A, lanes 4 and 8. When nuclear extracts of cells transfected with pcNet were incubated with pmE-b (mutated in EBS), the band disappeared (Fig. 4A, lanes 11 and 12). These results indicate that Net and Fli-1 bind to EBS in the pE probe. The star in Fig. 4A indicates three faster moving bands in lanes 3 and 4 that appeared when cells were transfected with pcNet. However, only the upper band among the three bands decreased its density after incubation with anti-V5 antibody. These results indicate that Net may form several types of DNA-protein complexes.

To examine the effects of Ets family proteins on the transcription of CTα, Ets family cDNAs were co-transfected with CTα promoter-luciferase construct LUC.C7 (−1268) (Fig. 4B). Luciferase activities were determined by dual-luciferase assays and normalized for transfection efficiency after co-transfection with pRL-TK Renilla vector. The Renilla luciferase activity of the thyminide kinase promoter-driven control was constant and was not significantly affected by Ets family proteins. Although Fli-1 binds to pE probe (Fig. 4A, lanes 7 and 8), there was not a significant change in luciferase activity by overexpression of Elk-1, ERF, Fli-1, or Tel compared with vector control as shown in Fig. 4B. Most surprisingly, when COS-7 cells were transfected with a cDNA encoding Net, the basal luciferase activity was almost completely inhibited (Fig. 4B). When COS-7 cells were transfected with various amounts of pcNet, the luciferase activities were decreased according to the amounts of the transfected plasmid and were significantly suppressed even with 0.005 μg of pcNet transfection (p < 0.005) (Fig. 4C). As shown in Fig. 4A, lane 3, Net may form a complex with some other proteins and pE. These results suggest that Net can act as an important repressor by interacting with binding proteins associated with the pE probe.

Because Ets-1 recognizes the same EBS as Net in pE, we were interested in whether or not the effects of Ets-1 on the luciferase activity were repressed by pcNet transfection in several cell lines (Fig. 5A and B). The
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expression of Net and Ets-1 was confirmed by Western blot analysis in COS-7 and NIH3T3 cells transfected with pcNet and pcEts-1. When cells were transfected with 0.25 μg of pcEts-1 and pcNet, proteins were equally expressed in COS-7 cells using anti-V5 antibody (data not shown). When cells were transfected with 0.25 μg of pcNet, overexpression of Ets-1 had little effect on the luciferase activity in both COS-7 and NIH3T3 cells (Fig. 5, A and B). Even when the cells were transfected with a small amount of pcNet (0.02 μg), Net significantly suppressed Ets-1-enhanced luciferase activity in both cell lines. These results suggest that the repressor effect of Net on CTα promoter may be more powerful than the activators, Ets-1.

Expression of Net Represses and RNAi Targeting Net Increases the Expression of the Endogenous mRNA for CTα in COS-7 and NIH3T3 Cells—We also determined if the expression of the endogenous CTα mRNA would be decreased in cells transfected with pcNet compared with pcDNA. To confirm the effects of Net on CTα transcription, we determined the amounts of mRNAs for CTα, Net, and G3PDH using quantitative real time RT-PCR. As shown in Fig. 5C, CTα mRNA decreased in response to the transfected amounts of pcNet (black bars). When cells were transfected with 1.25 μg of pcNet, the amount of endogenous CTα mRNA significantly decreased (p < 0.05).

We used Net-878 for RNAi experiments to confirm the effects of endogenous Net on CTα transcription. When Net-878 was transfected into NIH3T3 cells, Net mRNA content decreased to less than half compared with control after 24, 48, and 72 h of incubation (p < 0.05) (Fig. 5E). CTα mRNA significantly increased with Net-878, more than two times at 48 h compared with control (p < 0.05), and three times at 72 h (p < 0.01) (Fig. 5D). These results strongly suggested that Net could be a physiological suppressor of CTα transcription.

CTα, Ets-1, and Net mRNA Contents and Cellular Localization of Net in NIH3T3 Cells after Synchronization of the Cell Cycle—To clarify the physiological importance of Ets-1 and Net on CTα transcription in cell proliferation, the cell cycle was synchronized using NIH3T3 cells by 3 days of serum starvation, and was then followed by serum supplementation (Fig. 6). The amount of DNA increased 2-fold at 16 h, and the number of cells doubled by 24 h after serum supplementation (data not shown). Flow cytometric analysis showed that the S phase occurred between 12 and 16 h, and the M phase was completed by 24 h after serum supplementation (Fig. 6C). CTα mRNA in NIH3T3 cells significantly increased between 16 and 24 h compared with the level at 0 h (Fig. 6A). These results showed that CTα mRNA increased during the M phases in the cell cycle. As shown in Fig. 6B, the amount of Ets-1 mRNA significantly increased from 8 h after serum supplementation, and the increase was maintained after 24 h, and Net mRNA transiently increased at 8 h, and then returned to the base line. These results suggested that the increase of CTα mRNA may be due to the increase of Ets-1 mRNA during the cell cycle. At 8 h after serum supplementation, Ets-1 mRNA was increased but without an increase of CTα mRNA. This might be explained by the suppressive effects of Net because the mRNA for this factor was also increased at 8 h. After 16 h Net mRNA decreased to the baseline and then might lose its suppressive effects.

To certify the effects of Net on the cell cycle, pcNet was transfected into NIH3T3 cells when serum-starved cells were stimulated with 10% serum medium. Flow cytometric analysis showed that the cell number at G2/M phase significantly decreased when Net was overexpressed compared with vector control (17.48 ± 1.22% versus 21.04 ± 1.21% (n = 4); p < 0.03, respectively) at 24 h after transfection. These results suggest that Net may delay the cell cycle.

Some of Ets family proteins have been reported to translocate in cells and then control the transcription of their target genes. To determine the cellular localization of Net during the cell cycle, pEGFP-Net was transfected into NIH3T3 cells when serum-starved cells were stimulated with 10% serum medium. Flow cytometric analysis showed that the cell number at G2/M phase significantly decreased when Net was overexpressed compared with vector control (17.48 ± 1.22% versus 21.04 ± 1.21% (n = 4); p < 0.03, respectively) at 24 h after transfection. These results suggest that Net may delay the cell cycle.
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FIGURE 6. The amounts of CTα, Ets-1, and Net mRNA and cell cycle localization of GFP-Net in NIH3T3 cells during the cell cycle. A, CTα mRNA contents in NIH3T3 cells at the indicated time of serum supplementation (10% FBS) after 3 days of serum starvation (0.5% FBS) were quantified using DNA Engine Opticon 2 system. The values for GAPDH mRNA were used to normalize the CTα mRNA contents. Values are means ± S.D. from four independent dishes. Each experiment was repeated twice with similar results. * shows significant differences compared with 0 h (p < 0.02). B, Ets-1 (white bar) or Net (black bar) mRNAs in NIH3T3 cells at the indicated time after 3 days of serum starvation followed by serum supplementation were quantified using DNA Engine Opticon 2 system. The values for GAPDH mRNA were used to normalize the Ets and Net mRNAs. Values are means ± S.D. from four independent dishes. Each experiment was repeated twice with similar results. * shows significant differences compared with 0 h (p < 0.02). C, Flow cytometric analyses of the cell cycle for NIH3T3 cells at the indicated time after serum supplementations were performed. Each experiment was repeated twice with similar results. D, NIH3T3 cells without serum starvation were transfected with pEGFP-Net (0.5 μg) for 24 h, and fluorescent microscopic image for GFP-Net (×40) was obtained (left panel). Phase contrast image at the same position in left panel was shown (right panel). E, after 3 days of serum starvation (0.5% FBS), the medium was changed to 10% FBS, and then NIH3T3 cells were transfected with pEGFP-Net (0.5 μg) for 24 h, and fluorescent microscopic image for GFP-Net (×40) was obtained (left panel). Phase contrast image at the same position in left panel was shown (right panel). F, NIH3T3 cells without serum starvation were transfected with pEGFP-Netm1 (0.5 μg) for 24 h, and fluorescent microscopic image for GFP-Netm1 (×40) was obtained (left panel). Phase contrast image at the same position in left panel was shown (right panel). G, luc.C7 (0.5 μg), pRL-TK (0.001 μg), and pcDNA (0.25 μg) (white bar), pcNet (0.25 μg) (black bar), or pcNetm1 (0.25 μg) (oblique cross-striped bar) were transfected into NIH3T3 cells. Reporter activities were measured 24 h after transfection and normalized for transfection efficiency relative to pRL-TK. Values are means ± S.D. from four independent dishes. Each experiment was repeated three times with similar results.

expressed in NIH3T3 cells, the inhibitory effects of Net on luciferase activity were relieved compared with intact Net expression (p < 0.001) (Fig. 6G). These results suggest that nuclear localization of Net was important for suppressive effects of Net on CTα transcription.

DISCUSSION

Sp1 (at −58/−54) Interacts with Ets-1 (at −49/−47) and Co-activates Basal CTα Transcription—Ets protein was first identified as a product of one of three transforming genes in avian erythroblastosis virus E26 (32). The structures of Ets-1 and its isoform, Ets-2, were initially reported by Glyshdael et al. (33) and Watson et al. (34, 35). Ets-1 and Ets-2 are ubiquitous proteins that share significant homology (34, 35) and transcriptional activators that can be modulated by ras-dependent phosphorylation (36–38). Ets-1 and Ets-2 have three domains as follows: the C-terminal conserved Ets DNA binding domain (a winged helix-turn-helix), an N-terminal domain referred to as the “pointed domain,” and a trans-activation domain between these two structures (29). Eighteen Ets domain-containing genes have been identified in the human genome, and more than 200 promoters were reported to contain EBS (29, 30). Recently, several factors, such as NF-κB (39), core binding factor (40), and Sp1 (41), were reported to form complexes with Ets-1 and act as enhancers or modulators of DNA binding and transactivation by Ets-1 (42).

Cooperative interaction between Ets-1 and Sp1 for positive regulation was initially reported on the long terminal repeat of human T cell lymphotropic virus type 1 by Gegonne et al. (43). They suggested that Sp1/EBS elements were found in the promoter of several other cellular genes, and synergy between Ets-1 and Sp1 is not restricted to the long terminal repeat. Recently, Ets-1 and Sp1 were reported to cooperatively enhance the transcription of several genes for integrin αv (44), parathyroid hormone-related protein (41), protein kinase CK2α (45), and sarcoplasmic reticulum Ca2+-ATP type 3 (46). A physical association between Sp1 and Ets-1 was reported by Dittmer et al. (41) in the parathyroid hormone-related protein promoter through human T cell lymphotropic virus type 1 Tax protein, and in Fas ligand transcription by Kavurma et al. (47). Previously, we reported that Ets-1 binds to its GGA
These results indicate for the first time that Ets-1 interacts with Sp1 on Sp1. The importance of the Sp1-binding site (for cooperative activation of basal CT transcription of CT promoter, and both EBS and Sp1-binding sites are important for cooperative activation of basal CTα transcription. We thought that the slow moving bands indicated by arrow b in Fig. 1C, lane 1, consisted of the actual DNA-protein complex responsible for the activation of CTα transcription and may contain protein(s) in addition to Ets-1 and Sp1. The importance of the Sp1-binding site (−67/−62) for Ha-Ras stimulation (25) and Sp1 binding in the cell cycle (26) was observed in C3H10T1/2 cells. We postulate that both or either of −67/−62 and −58/−54 sites play important roles in regulating CTα transcription, depending on cell type or cellular status, and cooperative enhancement of basal CTα transcription by Ets-1 at −49/−47 and Sp1 at −58/−54 has significant potency in COS-7 and NIH3T3 cells.

Net Is a Suppressor of CTα Transcription—Although most of the known ETS proteins have been shown to activate transcription, four ETS proteins, Net, (48, 49), ERF (50), Fli-1 (31), and Tel (51), were reported as transcriptional repressors on several promoters. Therefore, we were interested in whether or not these four Ets family members have any transcriptional effects on Pcyt1A. As shown in this study, only Net exhibited a dramatic repressing activity on CTα transcription (Fig. 4B). Although ERF, Fli-1, and Tel have clear transcriptional suppressing activities on many promoters (31, 50, 51), they did not suppress CTα transcription. Giovane et al. (48) and Lopez et al. (49) reported that Net is composed of 409 amino acids, and its mRNA is expressed in most tissues and cells, although there is a significant variation in abundance (48). Net has several domains as follows: The N-terminal conserved ETS domain for DNA binding (domain A), A C-terminal domain phosphorylated by the Ras signaling pathway (domain C), and domain B interacting with SRF between these two structures (49). Most interestingly, Net contains two NLSs: one is located in domain A, and the other is in a fourth domain D near the N terminus of domain C, and one nuclear export signal is in domain A (52). Although Net protein mutated at domain D remained in the nucleus (52), homogeneous distribution of Net was observed with the mutation at NLS in domain A as shown in Fig. 6F. These results showed that NLS in domain A is more important for nuclear localization of Net than NLS in domain D.

Net has several homologous regions to Elk-1 and SAP-1, a subgroup of the Ets family proteins that interacts with SRF. Therefore, Net is recognized as one of the members of ternary complex factor and interacts with SRF on c-fos serum-response element (SRE) (48, 49, 53, and 54). Elk-1 and SAP-1 efficiently bind the c-fos SRE to enhance c-fos SRE reporter transcription. On the other hand, the ternary complex formation by Net was weak (53, 55); furthermore, its C-terminal sequence strongly inhibited the complex formation (53). The suppression of c-fos SRE-reporter transcription by Net was reported by Giovane et al. (55), whereas clear suppression was not observed by Price et al. (53). The other Net-binding site was reported in IgH enhancer π site, but the binding was also weak (49). Recently, Chen et al. (56) reported that Net is a strong repressor for the nitric-oxide synthase 2 promoter, and its expression is positively regulated by transforming growth factor-β1 and is negatively regulated by lipopolysaccharide in macrophages. They showed the physiological importance of Net but did not identify the binding site of Net in the promoter. Yamazaki et al. (57) first indicated a binding site for Net on the promoter of cytosolic chaperonin θ subunit independent of SRF, but they did not observe any difference between Net and Elk-1 on their transcriptional effects. As we report here, the NET-binding site on the CTα promoter is the first NET-binding site that does not contain an SRF binding consensus region (58). Furthermore, the same EBS in the CTα promoter is recognized by a complex of Ets-1 and Sp1. Net dramatically and dose-dependently suppressed Ets-1-enhanced CTα transcription (Fig. 5, A and B), whereas Elk-1 had no effect (Fig. 4B). As shown in Fig. 5, C and D, expression of Net decreased whereas RNAi targeting Net increased endogenous CTα mRNA. These results strongly suggest that Net participates in the regulation of the amount of endogenous suppression of CTα mRNA. Therefore, EBS (−49/−47) in the CTα promoter is a new transcriptional regulatory region recognized by Ets family proteins.

CTα mRNA expression (17), CTα activity, and PC biosynthesis (59, 60) increase between the G1 and the S phases of the cell cycle. To clarify the physiological importance of Ets-1 and Net on CTα transcription in cell proliferation, the cell cycle was synchronized by using NIH3T3 cells by 3 days of serum starvation and then serum supplementation. As shown in Fig. 6, A and B, Ets-1 and Net mRNA contents increased and decreased in NIH3T3 cells after serum supplementation as if they are regulating the CTα mRNA contents during the cell cycle. These results indicate that Ets-1 and Net are physiologically controlling CTα transcription during the cell cycle.

Ducret et al. (52) reported that a fusion protein of Net with green fluorescent protein is localized in the nucleus when it is expressed in cells, and its localization is changed to the cytoplasm in response to anisomycin, UV light, and heat shock mediated by c-Jun N-terminal kinase kinase. As shown in Fig. 6, D and E, GFP-Net was translocated from the nucleus to the cytoplasm 24 h after serum supplementation, although GFP-Net was mainly located in the nucleus under the standard culture conditions. These results indicate that the nuclear localization of Net is important for the suppressive effect of Net on CTα transcription. These results suggest that Net may move through the nuclear membrane, and nuclear exclusion would relieve transcriptional repression by Net. However, little is known about the precise signaling mechanism for nuclear exclusion of Net.

In this study, we showed that two Ets family proteins have different mechanisms in transcriptional regulation of CTα at the common EBS (−49/−47). Namely, Ets-1 makes a ternary complex with Sp1 and the promoter to enhance the transcription, and Net strongly inhibits the transcription even with the overexpression of Ets-1. Because these two Ets family proteins are distributed in many cells and tissues, their roles in regulation of CTα transcription might be important to understand the control of PC synthesis from a viewpoint of the cell cycle and cell proliferation.

Acknowledgments—We thank Drs. Kazuaki Tatei and Claudia Banchio for helpful comments on the manuscript.

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