Protein Kinase C-dependent, CCAAT/Enhancer Binding Protein β-mediated Expression of Insulin-like Growth Factor I Gene

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Running Title

C/EBPβ Mediates PKC Regulation of IGF-I Gene Transcription
Summary

The possible involvement of the protein kinase C (PKC) pathway in transcriptional regulation of the human insulin-like growth factor-I (IGF-I) gene has been suggested. In this study, we sought to determine whether a PKC-dependent pathway is implicated in the transcriptional control, and if it is, how this occurs. Treatment with 12-o-tetradecanoylphorbol 13-acetate (TPA) caused an increase in the activity of the human IGF-I gene major promoter in HepG2 cells. A CCAAT/enhancer binding protein (C/EBP) binding site located at +22 to +30 was bound by C/EBPβ in a TPA-dependent manner, and was solely responsible for the TPA responsiveness. This increase in C/EBPβ activity occurs through transcriptional and post-translational regulation and the latter is mediated by activation of p90 ribosomal S6 kinase (RSK): co-expression of dominant negative RSK abolished the TPA-responsive and C/EBPβ-dependent transactivation. Also, TPA-responsive activation of GAL4-C/EBPβ chimera required the Ser residue known as the RSK target. In SK-N-MC cells, which display constitutive, high expression of IGF-I on use of the major promoter, a large amount of C/EBPβ binding was observed with the C/EBP site in the basal state. Treatment with PKC inhibitors substantially reduced the promoter activity and mRNA amounts of IGF-I, with the binding of C/EBPβ to the C/EBP site also being reduced. When the C/EBP site was disrupted, the basal promoter activity was reduced but the reduction by the PKC inhibitor was no longer observed. These observations suggest that the increase of C/EBPβ binding to the C/EBP site, which is in part mediated via activation of RSK, can primarily explain the TPA responsiveness of the IGF-I gene promoter. The intrinsic PKC activity in SK-N-MC cells should play a major role in
the constitutive, high expression of IGF-I and may therefore contribute in part to the maintenance of the tumor phenotype of the cells.

*Key words:* IGF-I, Protein Kinase C, C/EBPβ, Ribosomal S6 Kinase, Gene Expression Regulation, Signal Transduction, Transcription Factors
Introduction

Insulin-like growth factor I (IGF-I)\(^1\), a 70-residue single-chain growth-promoting polypeptide, is produced in many organs and tissues and plays a major role in somatic growth, cell survival, tissue differentiation and intermediary metabolism (1-3). Although various tissue-dependent factors as well as endocrine hormones seem to regulate the IGF-I gene expression, their mechanisms, except for those involved in prostaglandin E\(_2\) or cAMP (4-6), are poorly understood.

The protein kinase-C (PKC) pathway is among the few that have been suggested to be involved in IGF-I gene regulation. The result of a nuclear run-on assay indicated that treatment of human macrophage-like cells with 12-o-tetradecanoylphorbol 13-acetate (TPA) increased the transcription rate of the IGF-I gene four- to fivefold (7), suggesting that the human IGF-I gene regulatory sequences contain something that responds to PKC. Support for this also comes from our recent observations with the chicken IGF-I gene, i.e., that the gene promoter can be activated by TPA through an AP-1 binding site located in it (8). However, it is unknown whether the mammalian IGF-I genes are activated by PKC, and if they are, how this occurs.

In contrast to protein kinase A, which seems to be involved in parathyroid hormone or prostaglandin E\(_2\)-induced IGF-I gene activation, PKC has been often discussed in correlation with tumorigenesis. Indeed, the best-known activator of PKC, TPA, is a strong tumor promoter (9,10). In vitro overexpression studies have suggested that individual PKC isozymes control cell proliferation and malignant transformation. For example, when PKC\(_\beta I\) was overexpressed in rat fibroblasts, the cells were partially transformed and could form tumors in nude mice (11). Overexpression of
PKCα also occasionally leads to transformation of fibroblasts (12). Because the IGF system is known to play an essential role in inducing transformation (13) or maintaining the tumor phenotype in some cells such as a human neuroblastoma cell line, SK-N-MC (14), it is likely that PKC-dependent activation of the IGF-I gene, if it occurs in mammals, may be partially involved in the tumorgenesis.

As a step toward elucidating the molecular basis of IGF-I gene regulation, we examined whether the human IGF-I gene promoter is a target of PKC regulation and sought to elucidate the physiological roles of the PKC pathway in the gene expression. Here we report that the major promoter of the gene, which is located within the 5′-flanking region and untranslated region of exon 1, is indeed a target of TPA stimulation and a CCAAT/enhancer binding protein (C/EBP) site within the promoter is responsible for the phenomenon. C/EBPβ, which is activated by PKC both at the level of transcription and of post-translation, binds to the C/EBP site and thereby mediates the phenomenon. Interestingly, the post-translational activation of C/EBPβ occurs primarily through activation of p90 ribosomal S6 kinase (RSK). Moreover, as support for the pathophysiological significance of these findings, we found that the constitutive IGF-I gene expression in the human neuroblastoma-derived SK-N-MC cells depends on the intrinsically activated PKC.
Experimental Procedures

**Antibodies**—Antibodies to C/EBPα (14AA), C/EBPβ (C-19), C/EBPδ (C-22), HNF-1α (C-19), and to c-Myb (M-19) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Antibodies to c-Fos and to c-Jun were purchased from Oncogene Science (Uniondale, NY). Antibody to HA-tag was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Cell Culture**—HepG2 cells (Riken Cell Bank, Tsukuba, Japan, catalog #RCB459) were maintained as previously described (15). SK-N-MC cells (ATCC Catalog No. HTB10) were maintained in EMEM supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids, penicillin and streptomycin (basal condition medium). 293T cells were maintained as previously described (16).

**Plasmids**—Human IGF-I promoter-1-luciferase fusion genes were constructed as follows. A phage clone containing the 5’-flanking region and exon 1 of the human IGF-I gene was isolated from a human genomic library and used as a template to make the reporter gene plasmids. A series of PCR was performed using the phage DNA as a template to amplify promoter-1 DNA fragments, which were comprised of either 1600 bp or 300 bp of the human IGF-I gene 5’-flanking region and the 197 bp of the exon 1 untranslated region. The PCR primers used were 5’-GCGGTACCAGCCTCTCAATGACACAATCTG-3’(for 1600 bp fragment), 5’-GCGGTACCAGTTGCTGGAGAGGGTCT-3’(for 300 bp fragment) and 5’-GGCAAGCTTGCGCAGGCTCTATCTGCT-3’(for both). To make the plasmid pIGFI-1600 (Fig. 1), the PCR-amplified 1600 bp fragment was made blunt-ended using the DNA Blunting Kit (Takara, Kyoto, Japan), digested with HindIII, and ligated into Smal/HindIII-digested pA3Luc (a kind gift from I.H. Maxwell, University of Colorado Health Science Center, Denver, CO) (17). The plasmid pIGFI-600 (Fig. 1)
was constructed by digesting the 1600 bp fragment with KpnI and HindIII, and subcloning the resulting 600 bp fragment into the KpnI/HindIII-digested pA3Luc. The 300 bp fragment was digested with KpnI and HindIII and ligated into KpnI/HindIII-digested pA3Luc to construct the plasmid pIGFI-300 (Fig. 1). Site-directed mutagenesis was performed as described previously (8).

C/EBPβ expression vector pcDNA3C/EBPβ has been described previously (5). Wild type (WT) and dominant negative (DN) type ribosomal S6 kinase (RSK) expression vectors were gifts from Dr. J. Blenis (Boston, MA). The plasmid pMSV β-gal is an expression plasmid of β-galactosidase gene driven by the murine sarcoma virus long terminal repeat (18). The plasmid pRL-CMV was purchased from Promega Corp (Madison, WI).

**Gene transfer experiments**—Transfection studies using HepG2 cell were performed as follows. One microgram of IGF-I promoter-1-luciferase fusion genes were co-transfected with 500 ng of pMSV β-gal to normalize for transfection efficiency. Cultures at 50% confluent density were rinsed in serum-free medium and exposed to plasmids in the presence of Lipofectamin™ for 5 h. After washing the plates two times with PBS, the solution was then replaced with serum-free medium (15), and the cells were incubated for 24 h. Next, the cells were treated for 24 h with vehicle (DMSO) or 10⁻⁷ M TPA. After incubation, the medium was aspirated, the cultures were rinsed with PBS twice and lysed in cell lysis buffer (Promega), and luciferase activity was measured as described previously (15).

Transfection studies using SK-N-MC cells were performed as follows. One microgram of IGF-I promoter-1-luciferase fusion genes were co-transfected with 5 ng of pRL-CMV to normalize for transfection efficiency. Cultures at 50% confluent density were rinsed in serum-free medium and exposed to plasmids in the presence of Lipofectamin™ for 5 h. After washing the plates two times with PBS, the
solution was replaced with culture medium containing 10% fetal bovine serum, and the cells were incubated for 24 h. Next, the cells were treated for 24 h with vehicle (DMSO) or $10^{-7}$ M GF109203X. After incubation, the medium was aspirated, the cultures were rinsed with PBS twice and lysed in cell lysis buffer, and dual-luciferase assay was performed according to the manufacturer's instructions (Promega).

Transfection studies using 293T cells were performed basically in the same way as the SK-N-MC cells described above. One micro gram of IGF-I promoter-1-luciferase fusion genes were co-transfected with indicated amount of C/EBPβ expression vector, 1 µg of wild type or dominant negative type RSK expression vector, when required, and 5 ng of pRL-CMV. After transfection, the cells were incubated for 24 h and dual-luciferase assay (Promega) was performed following the manufacturer's directions.

**Gal4 fusion protein reporter gene analyses**—The Gal4 fusion constructs (Gal4C/EBPβ118 and Gal4C/EBPβ166) were generated by isolating (by PCR) and introducing appropriate DNA fragments of C/EBPβ into the EcoRI-BglII site of pFACMV plasmid (Stratagene) which contained the DNA-binding domain (positions 1 to 147) of Gal4. Site-directed mutagenesis was performed with QuikChange Site-Directed Mutagenesis Kit (Stratagene) using two synthetic complementary oligonucleotides 5′-CCGAGCAAGAAGCCGCTACGGTTACG-3′ and 5′-CGTAACGTTACGGCTTGTGCTCGG-3′ (mutated sequence was underlined) to generate Gal4C/EBPβ118mutAla105 and Gal4C/EBPβ166mut105Ala. The Gal4-responsive reporter plasmid pFR-Luc plasmid containing 5 copies of Gal4-binding element upstream of the basic promoter element (TATA box) linked to luciferase structural gene was purchased (Stratagene). By lipofection, 1 µg of each Gal4 fusion plasmid was cotransfected into
host cell with 1 µg of pFR-Luc and 5 ng of pRL-CMV. The cells were then incubated for 48 h, followed by a dual-luciferase assay (Promega) performed according to the manufacturer’s directions.

**Nuclear Protein Extracts**—HepG2 and SK-N-MC cell nuclear extracts were prepared by the method of Lee et al. (19) with minor modifications. Cells were harvested with a cell scraper and gently pelleted, and the pellets were washed with phosphate-buffered saline. The cells were then lysed in hypotonic buffer (10 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol). Nuclei were pelleted and resuspended in hypertonic buffer containing 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, and 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. Soluble proteins released by a 30-min incubation at 4°C were collected by centrifugation at 12,000 x g for 20 min, and the supernatant was collected. The protein concentration was measured using a modified Bradford assay (Bio-Rad).

**DNA-Protein Binding Studies**—Gel mobility shift experiments followed previously published methods (4). Radiolabeled double-stranded DNA probes were synthesized by annealing complementary end-labeled oligonucleotides. Nuclear protein extracts (5 µg) were preincubated for 20 min on ice with 2 mg of poly(dI-dC) with or without unlabeled specific or nonspecific DNA competitor or antibodies in 25 mM HEPES, pH 7.6, 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.025% bovine serum albumin. After the addition of 5 x 10⁴ cpm of DNA probe for 30 min on ice, the samples were applied to 12% nondenaturing polyacrylamide gel that had been pre-electrophoresed for 30 min at 12.5 V/cm at 25 C in 45 mM Tris, 45 mM boric acid, 1 mM EDTA. Electrophoresis was conducted for 2.5 h under identical conditions. The dried gels were exposed to x-ray film at –80 C with an intensifying screen.
Deoxynuclease I (DNase) footprinting was performed as described elsewhere (6). End-labeled double-stranded DNA probes flanking the C/EBP site in human IGF-I promoter-1, which corresponds to -46 to +96 bp (relative to transcriptional start site) of the human IGF-I gene, were generated by polymerase chain reaction, using one end-labeled oligonucleotide primer (5’-ATGCTCTGTCTCTAGTT-3’) and one unlabeled primer (5’-ACTGTAGACAGGAAACAGCT-3’). Nuclear protein (10 µg) was preincubated for 15 min with poly(dI·dC) in 25 mM HEPES, pH 7.6, 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.05% bovine serum albumin, followed by the addition of labeled probe (5.0 x 10⁵ cpm/sample) and incubation for 60 min on ice. The reaction mixture was then treated with DNase I (final concentration 1.15 mg/ml, Worthington Biochemical Corp., Freehold, NJ) in 2.5 mM MgCl₂ and 2.5 mM CaCl₂ for 1 min at 25 C. Nuclease treatment was terminated by addition of 20 mM EDTA, 200 mM NaCl, 1% sodium dodecyl sulfate, and 10 mg of yeast transfer RNA, followed by phenol-chloroform extraction and ethanol precipitation. Samples were analyzed after electrophoresis on 8% polyacrylamide, 8 M urea gel and autoradiography for 16 h at –80 C with an intensifying screen.

**RNA Isolation and Analyses**—Total RNA was extracted from HepG2 cells or SK-N-MC cells by homogenization in guanidine thiocyanate. Northern blots followed standard procedures using 10 µg of total RNA, and the buffer conditions were as described. The hybridization probes were 7 x 10⁶ cpm of ³²P-labeled rat C/EBPβ cDNA probe and human IGF-I cDNA probe. Reverse transcription (RT)-PCR were performed using primers 5’-ATCAGCGTCTTCCAACCCAATTA-3’ and 5’-TGGCGCTGGGCACGGACAGA-3’ (for human IGF-I) and 5’-AAGGCCGGCTTCGCGGCGA-3’ and 5’-CCGGCCAGCCAGGTCCAGAC-3’ (for β-actin).
Western Blot Analysis—293T cell nuclear proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After the membranes were blocked with 5% nonfat dry milk and 2% fetal bovine serum in 20 mM Tris-Cl, pH 7.6, 137 mM NaCl for 1 h at 25 C, they were incubated with an antibody to HA for 1 h at 25 C. Subsequent steps were performed as described elsewhere (5).
Results

Human IGF-I gene promoter-1 is activated by phorbol ester — First we examined whether the major promoter (promoter-1) of the human IGF-I gene is a target of PKC activation. A series of gene transfer studies were performed with human hepatocellular carcinoma-derived HepG2 cells. Under the basal condition, HepG2 cells barely express the IGF-I gene according to RT-PCR results but the IGF-I mRNA derived from the promoter 1 was induced when the cells were treated with $10^{-7}$ M TPA for 4 h (data not shown). Each reporter gene plasmid contained various lengths of IGF-I gene 5′-flanking sequences and 197 bp of the exon 1 untranslated region linked to the firefly luciferase reporter, because this portion of exon 1 untranslated region appeared to be important for basal promoter activity of promoter-1 in SK-N-MC cells (20).

As shown in Fig. 1, despite differences in the basal promoter activities, the promoter activities of the 1600 bp (pIGFI-1600), 600 bp (pIGFI-600), and 300 bp (pIGFI-300) were activated to a similar extent after treatment with TPA; about 2.5-fold. This result showed that the human IGF-I gene promoter-1 could be activated by TPA treatment of the cells and that the major portion of the cis-active elements mediating this phenomenon is located within the 300 bp of the 5′-flanking sequence and/or the 197 bp of the untranslated region of exon 1.

Identification of a putative TPA-responsive element — There was no region that perfectly matched the consensus AP-1 motif (TG/TAGTCA) within the region of the IGF-I gene which revealed the TPA responsiveness (-300 ~ +197). However, a region of high similarity to the AP-1 consensus was seen within the exon 1
untranslated region (+23 ~ +29; TTACTCA); indeed, the same sequence in the JE-1 gene was shown to be a target for TPA-responsive activation in MC3T3-E1 cells (21).

To find whether this portion is involved in the TPA responsiveness, we performed a mutation analysis. Because this portion was located within a region where multiple transcription initiation sites are clustered, it seemed possible that a mutation in this region could cause unpredictable, non-specific damage to the promoter activity. To avoid this, we changed one sequence of the possible TPA-responsive region ("A"(+29) to "G") so that the sequence became the same as the homologous regions of the chicken and rat IGF-I genes (22,23).

As shown in Fig. 1, when one nucleotide mutation was introduced into the portion (pIGFI-300M), TPA-induced promoter activation was completely abolished, showing that this portion does play an essential role in mediating TPA effects on the IGF-I gene promoter. The mutated promoter also caused a decrease in the basal promoter activity (Fig. 1).

\textbf{C/EBPβ binds to the putative TPA-responsive region} — Interestingly, the putative TPA-responsive region in the human IGF-I gene also contains the consensus for the C/EBP binding motif, CTTACTCAA. Indeed, Nolten et al. previously demonstrated in vitro that C/EBPα and C/EBPβ, when overexpressed, can bind to this region (24).

To characterize the factors involved in the TPA activation of the human IGF-I gene, we performed gel-mobility shift analyses. As shown in Fig. 2, TPA enhanced specific protein bindings to the putative TPA-responsive region (lanes 1 and 5). The unlabeled wild-type competitor but not the mutated competitor to which the same
point mutation was introduced as in the reporter gene construct ("A"(+29) to "G"), inhibited the DNA-protein bindings (lanes 6-11). Also, when the mutation was introduced to the labeled probe, no binding was observed at all (lane 12). Thus, a certain factor or factors bind to the putative TPA-responsive region in the human IGF-I gene promoter in a TPA-responsive manner and this mediates the TPA responsiveness of the gene transcription.

As mentioned above, the putative TPA-responsive region reveals similarity to the AP-1 motif but also is a potential C/EBP binding site. To identify the factor that mediates the TPA responsiveness by binding to the region, we next performed a gel mobility-supershift assay using specific antibodies against C/EBPα, C/EBPβ, C/EBPδ, c-Fos, c-Jun, HNF-1α and c-Myc, respectively. Each antibody was added to a tube prior to the binding reaction. As shown in Figure 3, a supershifted band was observed together with a reduction of the gel-shift complex only when the C/EBPβ antibody was added (lane 9). Thus, the results clearly indicated that the TPA-responsive binding protein includes C/EBPβ and the TPA-responsive region works as a C/EBP binding site.

Next, we performed a DNase footprinting assay with end-labeled double-stranded DNA probes derived from human IGF-I promoter-1 and nuclear extracts from HepG2 cell or C/EBPβ overexpressing 293T cells (Fig. 4). The results indicated that overexpressed C/EBPβ protected the C/EBP site from nuclease digestion (lanes 4 and 5). With the nuclear extract of HepG2 cell, the same site was protected (lane 2), and this protection was enhanced by 4 h incubation with TPA (lane 3), confirming that C/EBPβ binds to the C/EBP site in a TPA-dependent manner in HepG2 cells.
Thus, C/EBPβ binds to the C/EBP site in IGF-I gene promoter-1 and mediates the TPA effects on the IGF-I gene promoter.

**C/EBPβ transactivates IGF-I gene promoter-1** — To investigate whether C/EBPβ that binds to the C/EBP site can activate the IGF-I gene transcription, we overexpressed C/EBPβ in 293T cells and evaluated the effects on the IGF-I gene promoter activity. 293T cells were chosen because they lack intrinsic expression of C/EBPβ (data not shown). As shown in Fig. 5, overexpression of C/EBPβ transactivates the IGF-I promoter-1 in a dose-dependent manner. When a point mutation was introduced to the C/EBP site, the transactivation effect of C/EBPβ disappeared, suggesting that C/EBPβ transactivates human IGF-I promoter-1 through the C/EBP site. Taken together, these results indicate that PKC activation induces human IGF-I gene transcription through enhancement of the C/EBPβ binding to promoter 1.

**PKC transcriptionally activates C/EBPβ in HepG2 cells** — Next we investigated the mechanism that underlies the PKC-dependent activation of C/EBPβ. First, the effects of TPA on C/EBPβ mRNA were evaluated in HepG2 cells. The results of Northern blot analysis (Fig. 6) revealed that TPA stimulation increases the C/EBPβ mRNA amount by approximately 3-fold, suggesting that PKC can stimulate C/EBPβ gene transcription and this may in part explain the PKC activation of the human IGF gene promoter.

**PKC post-translationally activates C/EBPβ via activation of RSK** — Recently, RSK was shown to stimulate C/EBPβ activity and this facilitates TGFβ-induced hepatocyte proliferation (25). Because RSK is known to be a downstream target of
PKC, we investigated the possibility of RSK also being involved in the PKC-dependent activation of C/EBPβ and IGF-I gene activation. For this purpose, we used a dominant negative (DN) mutant of RSK.

As shown in Fig. 7, the DN RSK mutant, when co-overexpressed in the 293T cells with C/EBPβ, significantly suppressed the transactivation potential of C/EBPβ in terms of the activation of the human IGF-I gene promoter. This effect was not observed when C/EBPβ was absent (Fig. 7). These results suggest that the transactivation potential of C/EBPβ depends on the RSK activity in 293T cells.

To further clarify the mechanism underlying this RSK dependent activation of C/EBPβ, we employed the *Saccharomyces cerevisiae* GAL4 fusion protein reporter system (Fig 8). It is known that the N-terminal region of C/EBPβ contains a transcription activation domain and a transrepression domain (Fig. 8b, (26)). It also includes a Ser residue at 105, which was previously shown to be critical for the C/EBPβ activation by TPA (27) and was also identified recently as the phosphorylated site by RSK (25).

Accordingly, we prepared GAL4 fusion constructs containing either 118 or 166 amino acids (aa) of N-terminal region of C/EBPβ fused to the heterologous DNA-binding domain of the GAL4 transcription factor (Fig. 8b). These chimeric GAL4-C/EBPβ fusion proteins were expressed in HepG2 cells and effects on the GAL4 reporter were evaluated. HepG2 cells were used for this experiment because they show a very good response to TPA and have intrinsic C/EBPβ.
As shown in Fig. 8a (lane 3), the Gal4C/EBPβ118 construct transactivated the GAL4 reporter in serum-free medium. On the other hand, the Gal4C/EBPβ166 construct did not activate the GAL4 reporter in the basal state (lane 5). This observation is consistent with former report by Williams et al. (26) and provides further support for the idea that the transrepression domain was potent enough to almost totally suppress the transactivation potential. Unlike the Gal4C/EBPβ118 construct revealing no further activation by TPA (Fig. 8a; lanes 3 and 4), the Gal4C/EBPβ166 construct was converted to a transcriptional activator by the TPA treatment (lanes 5 and 6). This clearly indicated that TPA activates C/EBPβ by inhibiting the activity of transrepression domain rather than activating the transactivating domain.

In support of the implication of RSK, overexpression of DN RSK mutant abolished the TPA responsive activation of Gal4C/EBPβ166 (Fig. 8a; lanes 9 and 10). Moreover, when the previously identified phosphorylation site for RSK within C/EBPβ (Ser-105; (25)) was disrupted (substituted by Ala) in the GAL4 fusion protein (Gal4C/EBPβ166mut105Ala), it could not transactivate the GAL4 reporter in response to the TPA stimulation (lanes 11 and 12). These observations thus suggest that TPA stimulates activation function of C/EBPβ via RSK activation followed by the phosphorylation of Ser-105 of C/EBPβ.

We next investigated the implication of the RSK-C/EBPβ axis in the IGF-I gene regulation. As shown in Fig. 9, the coexpression of DN RSK mutant in TPA-stimulated HepG2 cells suppressed the promoter activity of the IGF-I gene in the cells. This agrees with the idea that RSK plays a primary role in mediating the TPA-dependent activation of C/EBPβ and, subsequently, the activation of the IGF-I
gene transcription. Taken together, these data demonstrated that PKC activates C/EBPβ both at the level of its transcription (Fig. 6) and of its post-translation, which is mediated by the activation of RSK (Figs. 7-9).

**IGF-I gene expression in SK-N-MC cells depends on PKC-dependent, transcriptional and post-translational regulation of C/EBPβ**— SK-N-MC is a human neuroblastoma cell line which displays constitutive expression of the IGF-I gene (22). It was previously shown that IGF-I mRNAs expressed in SK-N-MC cells contained exon 1 sequences but lacked exon 2 sequences and that promoter-1, but not promoter-2, was active in the cells (28). As support for physiological implication of the PKC-dependent pathway in the IGF-I gene regulation, we found that the IGF-I mRNA amount was significantly decreased in SK-N-MC cells when the cells were incubated for 24 h in the presence of the PKC inhibitor staurosporin (Fig. 10).

To clarify the factors involved in the intrinsic IGF-I gene expression in SK-N-MC cells, we investigated whether a certain nuclear factor binds to the C/EBP site of the human IGF-I gene in SK-N-MC cells. According to results of gel-mobility shift assays (Fig. 11), there was a nuclear protein that specifically binds to the C/EBP site. This agreed with the results of the DNase footprinting assay shown in Fig. 4 (lane 6). A group of specific antibodies were added to identify the binding protein and the results clearly demonstrated that only C/EBPβ occupies the C/EBP site in SK-N-MC cells (Fig. 11; lanes 8-14). Because the intensity of the retarded band (C/EBPβ) was weakened when a PKC inhibitor staurosporin was added to the cells (lanes 4-6), we concluded that PKC, which seems readily activated even in the basal state (without any stimulation) in SK-N-MC cells, is involved in the activation of the nuclear protein binding to the C/EBP site in these cells.
We next sought to determine whether this PKC-dependent binding of C/EBPβ to the C/EBP site is required for the human IGF-I gene expression in SK-N-MC cells. Results of reporter gene analyses revealed that incubation of SK-N-MC cells with a specific PKC inhibitor GF109203X decreased the basal promoter activity of 1600-bp (pIGFI-1600), 600-bp (pIGFI-600), and 300-bp (pIGFI-300) human IGF-I promoter-1 by more than 50% (Fig. 12). This suppressive effect of the PKC inhibitor was no longer observed with pIGFI-300M in which the C/EBP site was disrupted (Fig. 12), suggesting that intrinsically activated PKC enhances the C/EBPβ-binding to the C/EBP site and thus is responsible for the human IGF-I gene transcription.

To investigate whether both transcriptional and post-transcriptional regulation of C/EBPβ by PKC is involved in IGF-I gene expression in SK-N-MC cells, we performed Northern blot analyses and GAL4 fusion protein reporter analyses. As shown in Fig. 13a, the amount of C/EBPβ mRNA was decreased in SK-N-MC cells after a 24-h incubation with staurosporin, suggesting that the transcriptional control of C/EBPβ by intrinsically activated PKC can in part be involved in the regulation of IGF-I gene expression in SK-N-MC cells.

On the other hand, the GAL4 fusion protein reporter assays in SK-N-MC cells revealed that the Gal4C/EBPβ166 construct transactivated the GAL4 reporter in SK-N-MC cells kept under the basal condition (Fig. 13b; lane 2). This contrasted with the observation obtained with HepG2 cells, in which TPA was required for the transactivation potential activated (Fig. 8; lanes 5 and 6). The transactivation potential was dramatically suppressed by addition of PKC inhibitor GF10923X (Fig.
13b: lane 3), suggesting that intrinsically activated PKC readily activated C/EBPβ in SK-N-MC cells even without any stimuli. Substitution of Ser-105 by Ala (Gal4C/EBPβ166mut105Ala) or overexpression of the DN RSK mutant also suppressed the activation function of C/EBPβ (lane 4-6). Thus it was demonstrated that the IGF-I gene expression in SK-N-MC cells depends on the intrinsically activated PKC, which is involved in the activation of C/EBPβ probably through two different mechanisms: the increment of its transcription and the RSK-mediated post-translational activation of its transactivation potential.
In the present study, we showed that the major promoter of the human IGF-I gene is a target of TPA stimulation (Fig. 1) and a C/EBP site within the promoter is responsible for the phenomenon (Figs. 2-5). C/EBPβ, which is activated by PKC both at the level of transcription (Figs. 6 and 13a) and of post-translation (Figs. 8 and 13b), binds to the C/EBP site and thereby mediates the phenomenon. In non-C/EBPβ-expressing 293T cells, transiently expressed ectopic C/EBPβ transactivated the human IGF-I gene promoter (Figs. 5 and 7) and indeed induced intrinsic IGF-I gene expression in the cells (Y.U. and Y.K.; unpublished observation), providing support for the physiological implication of C/EBPβ in the IGF-I gene expression.

The C/EBP family proteins comprise a diverse group of transcriptional regulators active in tissue development and regeneration, inflammation, and intermediary metabolism (29,30). They are members of the basic leucine zipper family of transcription factors (29-31) and also reveal strong amino acid similarity in their COOH-domains, which have been shown to be responsible for protein dimerization and DNA binding (29,30).

Nolten et al. (24) previously reported that overexpressed C/EBPα and C/EBPβ can bind to the region that we found to be the putative TPA-responsive region and can stimulate the promoter activity of human IGF-I promoter-1. Our data showed that, at least in the case of HepG2 and SK-N-MC cells, C/EBPβ is the major binding factor for the C/EBP site and that no other C/EBP family proteins such as C/EBPα and C/EBPδ are included in the binding complexes formed with the C/EBP site of the human IGF-I gene promoter. Whereas the C/EBP family proteins share a
common binding preference, this may be because the C/EBP family proteins other than C/EBPβ are not expressed in those cells or because they are inactivate even when expressed.

The human neuroblastoma cell line SK-N-MC reveals relatively high expression of IGF-I under the basal condition; the amount of IGF-I mRNA was even more than that expressed in the human liver (22). The fact that the addition of neutralized antibody to IGF-IR prevents the cell from proliferating (14), suggests that the IGF-I autocrine loop is essential for the tumor phenotype of SK-N-MC cells. As a step toward elucidating the mechanism underlying the constitutive expression of IGF-I gene in SK-N-MC cells, we sought to clarify the physiological role of the C/EBP site in the IGF-I gene expression. The results of the DNase footprinting assay (Fig. 4) and supershift assay (Fig. 11) clearly showed that C/EBPβ bind to the C/EBP site in SK-N-MC cells under basal condition, and reporter gene analysis (Fig. 12) showed that the C/EBPβ binding to the C/EBP site is crucial for strong activity of the promoter-1 in SK-N-MC cells. Support for this comes from the observation by Mittanck et al. (19) showing that the initial 50 base pairs of the exon 1 untranslated region are essential for the promoter-1 of human IGF-I gene being active in the SK-N-MC cell line, although, for some unknown reason, they failed to detect any protein binding with a probe containing the C/EBP site.

The fact that the mutated human C/EBP site we used in this study, whose sequence completely matches the sequence of the corresponding region of rat IGF-I promoter-1, could not be bound by C/EBPβ, clearly showed that this C/EBP site is not conserved in rat or mouse IGF-I gene. However, we previously identified another high affinity C/EBP site, termed the HS3D site, in rat IGF-I promoter-1 located 5'.
untranslated region of exon 1 (4-6). As support for the extensive implication of C/EBP family proteins in IGF-I gene regulation, another C/EBP family protein, C/EBPδ, was shown to mediate cAMP responsiveness of rat IGF-I promoter-1 through this site (5,6). Considering the conserved binding preference for the C/EBP family proteins, it is possible that C/EBP family proteins are involved in various regulation of the IGF-I gene through the same C/EBP binding site with their activity being post-translationally modulated by various kinases.

A serine/threonine kinase RSK has been shown to function as a downstream target of PKC (32,33). Tan et al. (32) showed that RSK mediates the PKC-dependent prevention of Bad-mediated apoptosis by directly phosphorylating Bad protein. In terms of the substrates of RSK, only a few have been identified to date including transcription factors CREB and Fos (34,35). Recently, C/EBPβ was added to the list of direct substrates of RSK: activated RSK phosphorylates Ser-105 of C/EBPβ and this activation is critical for hepatocyte proliferation (25). These reports prompted us to consider that RSK may mediate the present phenomenon: using a dominant negative mutant of RSK, we were able to show that RSK plays a key role in the PKC-dependent, C/EBPβ-mediated activation of the IGF-I gene promoter (Figs. 7-9). Moreover, the data obtained with Saccharomyces cerevisiae GAL4 fusion protein reporter system revealed that the TPA-responsive activation of the GAL4-C/EBPβ chimera depends on a Ser residue at position 105 (Ser-105) of C/EBPβ (Fig. 8). Because Ser-105 is known as a direct phosphorylation site by RSK (25), it is likely that the TPA-induced transactivation of C/EBPβ is also mediated by direct phosphorylation/post-translational activation by RSK.
Although PKC seems to act as an essential upstream factor for the RSK-dependent C/EBPβ-mediated IGF-I gene activation in SK-N-MC cells, this may not be the case for all cell types. For example, in spite of the implication of RSK activation in the C/EBPβ-mediated IGF-I gene activation in 293T cells (Fig. 7), there has been no evidence reported to date that supports the constitutive PKC activation in the cells. Rather, according to a report by Arould et al. (36), they directly measured PKC activity in 293T cells but detected no significant PKC activity, thus disputing the implication of PKC in the RSK activation in 293T cells. Although speculative, mitogen-activated protein kinases (MAPK), instead of PKC, may play a key role in the RSK activation in 293T cells. It was shown previously that MAPK can directly activate RSK (37). Also, 293T cell was transformed with SV40 large T antigen and E1A, both of which are well known activators for the MAPK cascade (38). Indeed, 10% serum stimulation was shown to cause a partial nuclear translocation of MEK in 293T cells (39). Because our 293T cells were always maintained in serum (10%)-containing medium, it is possible that the MAPK cascade was kept activated in the cells and thereby involved in the RSK activation. Thus, the RSK-C/EBPβ-IGF-I axis, which is probably activated not only by PKC but also by some growth factors that can activate MAPK, may operate in various cell types and contribute to the expression of IGF-I gene in those cells.

Apart from this RSK-mediated effect on the activation function of C/EBPβ, our present study failed to clarify whether the TPA treatment enhances the DNA-binding affinity of C/EBPβ. Although we found that TPA stimulation enhanced the C/EBPβ binding to its target DNA in HepG2 cells (Figs. 2 and 3), this does not necessarily mean that the DNA-binding affinity was increased in response to TPA as the increment of the C/EBPβ gene expression can by itself explain the
phenomenon (Fig. 6). Indeed, Trautwein et al. (26) previously reported that TPA stimulates transactivation potential of C/EBPβ without changing its DNA-binding affinity in HepG2 cells. Thus we assume that the RSK-mediated regulation of C/EBPβ activity takes place probably independent of controlling its DNA-binding affinity.

IGF-I is known to function as an autocrine or paracrine growth factor in a variety of mesenchymal and epithelial tumors (40). Recent studies have demonstrated that expression of the type-I insulin-like growth factor receptor (IGF-IR), which mediates most of the IGF-I action, is required for the establishment and maintenance of the transformed phenotype in some cell lines (13). A gene-targeting study revealed that cells derived from IGF-IR(-/-) mouse embryos can not be transformed by the SV40 large T antigen or by an activated and overexpressed Ha-ras, while stable transformation of cells with human IGF-IR expression plasmid restored the ability to be transformed (13). These findings suggest that the IGF system (including IGF-I(&IGF-II) and IGF-IR) plays an essential role in, at least, some cases of transformation or tumorigenesis. On the other hand, it is well-known that the PKC activator TPA is a strong carcinogen. Because the IGF-I gene expressed in SK-N-MC cells was shown to play a major role in the proliferation of SK-N-MC, it is likely that the constitutive active PKC subtypes contribute toward keeping the IGF system active and thereby maintaining the tumor phenotype of the cells.

In conclusion, our present study revealed that PKC can activate the IGF-I gene expression through the RSK and C/EBPβ-mediated pathway. Whereas the IGF-I system is essential for proliferation of SK-N-MC cells (14), constitutively active
PKC may contribute to determining tumor phenotype in some cancer cells by keeping the IGF-I gene expression active.
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References

1. Jones, J. I., and Clemmons, D. R. (1995) *Endocr Rev* **16**, 3-34.
2. Stewart, C. E., and Rotwein, P. (1996) *Physiol Rev* **76**, 1005-1026.
3. Baserga, R., Hongo, A., Rubini, M., Prisco, M., and Valentinis, B. (1997) *Biochim Biophys Acta* **1332**, F105-126.
4. Thomas, M. J., Umayahara, Y., Shu, H., Centrella, M., Rotwein, P., and McCarthy, T. L. (1996) *J Biol Chem* **271**, 21835-21841.
5. Umayahara, Y., Ji, C., Centrella, M., Rotwein, P., and McCarthy, T. L. (1997) *J Biol Chem* **272**, 31793-31800.
6. Umayahara, Y., Billiard, J., Ji, C., Centrella, M., McCarthy, T. L., and Rotwein, P. (1999) *J Biol Chem* **274**, 10609-10617.
7. Nagaoka, I., Trapnell, B. C., and Crystal, R. G. (1990) *J Clin Invest* **85**, 448-455.
8. Kajimoto, Y., Kawamori, R., Umayahara, Y., Iwama, N., Imano, E., Morishima, T., Yamasaki, Y., and Kamada, T. (1993) *Biochem Biophys Res Commun* **190**, 767-773.
9. Blobe, G. C., Obeid, L. M., and Hannun, Y. A. (1994) *Cancer Metastasis Rev* **13**, 411-431.
10. Gomez, D. E., Skilton, G., Alonso, D. F., and Kazanietz, M. G. (1999) *Oncol Rep* **6**, 1363-1370.
11. Housey, G. M., Johnson, M. D., Hsiao, W. L., O’Brien, C. A., Murphy, J. P., Kirschmeier, P., and Weinstein, I. B. (1988) *Cell* **52**, 343-354.
12. Megidish, T., and Mazurek, N. (1989) *Nature* **342**, 807-811.
13. Baserga, R. (1999) *Exp Cell Res* **253**, 1-6.
14. Kiess, W., Koepf, G., Christiansen, H., and Blum, W. F. (1997) Regul Pept 72, 19-29.
15. Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. (1994) J Biol Chem 269, 16433-16442.
16. Fujitani, Y., Kajimoto, Y., Yasuda, T., Matsuoka, T. A., Kaneto, H., Umayahara, Y., Fujita, N., Watada, H., Miyazaki, J. I., Yamasaki, Y., and Hori, M. (1999) Mol Cell Biol 19, 8281-8291.
17. Maxwell, I. H., Harrison, G. S., Wood, W. M., and Maxwell, F. (1989) Biotechniques 7, 276-280.
18. Rosenthal, N. (1987) Methods Enzymol 152, 704-720
19. Lee, K. A., Bindereif, A., and Green, M. R. (1988) Gene Anal Tech 5, 22-31.
20. Mittanck, D. W., Kim, S. W., and Rotwein, P. (1997) Mol Cell Endocrinol 126, 153-163.
21. Koike, M., Kuroki, T., and Nose, K. (1993) Mol Carcinog 8, 105-111
22. Kajimoto, Y., and Rotwein, P. (1991) J Biol Chem 266, 9724-9731.
23. Hall, L. J., Kajimoto, Y., Bichell, D., Kim, S. W., James, P. L., Counts, D., Nixon, L. J., Tobin, G., and Rotwein, P. (1992) DNA Cell Biol 11, 301-313.
24. Nolten, L. A., van Schaik, F. M., Steenbergh, P. H., and Sussenbach, J. S. (1994) Mol Endocrinol 8, 1636-1645.
25. Buck, M., Poli, V., van der Geer, P., Chojkier, M., and Hunter, T. (1999) Mol Cell 4, 1087-1092.
26. Williams, S. C., Baer, M., Dillner, A. J., and Johnson, P. F. (1995) Embo J 14, 3170-3183.
27. Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M., and Chojkier, M. (1993) Nature 364, 544-547.
28. Kim, S. W., Lajara, R., and Rotwein, P. (1991) Mol Endocrinol 5, 1964-1972.
29. Wedel, A., and Ziegler-Heitbrock, H. W. (1995) Immunobiology 193, 171-185.
30. Lekstrom-Himes, J., and Xanthopoulos, K. G. (1998) J Biol Chem 273, 28545-28548.
31. Habener, J. F., Miller, C. P., and Vallejo, M. (1995) Vitam Horm 51, 1-57
32. Fisher, T. L., and Blenis, J. (1996) Mol Cell Biol 16, 1212-1219.
33. Tan, Y., Ruan, H., Demeter, M. R., and Comb, M. J. (1999) J Biol Chem 274, 34859-34867.
34. Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) Science 273, 959-963.
35. Chen, R. H., Juo, P. C., Curran, T., and Blenis, J. (1996) Oncogene 12, 1493-1502.
36. Arnould, T., Kim, E., Tsiokas, L., Jochimsen, F., Gruning, W., Chang, J. D., and Walz, G. (1998) J Biol Chem 273, 6013-6018.
37. Blenis, J. (1993) Proc Natl Acad Sci U S A 90, 5889-5892.
38. Kim, K., Nose, K., and Shibanuma, M. (2000) J Biol Chem 275, 20685-20692.
39. Jaaro, H., Rubinfeld, H., Hanoch, T., and Seger, R. (1997) Proc Natl Acad Sci U S A 94, 3742-3747.
40. Macaulay, V. M. (1992) Br J Cancer 65, 311-320.
Footnotes

1. **Abbreviations used in this paper**: IGF-I, insulin-like growth factor-I; PKC, protein kinase C; TPA, 12-o-tetradecanoylphorbol 13-acetate; C/EBP, CCAAT/enhancer binding protein; RSK, p90 ribosomal S6 kinase; EMEM, Earle's modified Eagle's medium; IGF-IR, type-I insulin-like growth factor receptor; RT, reverse transcription; aa, amino acid(s); DBD, DNA-binding domain; MAPK, mitogen-activated protein kinases.
Figure Legends

Fig. 1. **Putative TPA responsive site is located in the 5’-untranslated region of exon 1 of human IGF-I gene.** Left panel, Diagrammatic representation of IGF-I promoter-1-luciferase reporter plasmids. The nucleotide sequence is shown for the region containing AP-1-like sequence (bold) and C/EBP binding site (underlined). pIGFI-300M has a point mutation and the altered base is in outline lettering. Right panel, IGF-I promoter-1-luciferase reporter plasmids were cotransfected with a pMSV-β-galactosidase control vector into HepG2 cell using Lipofectamin. After 24 h incubation, the cultures were exposed to control medium (containing vehicle) or TPA (100 nM) for 24 h. Cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency (β-galactosidase expression) and presented as means ± SD of at least three independent experiments performed in duplicate.

Fig. 2. **TPA induced specific nuclear protein binding to the putative TPA responsive site.** Gel mobility shift experiments were performed as described under “Experimental Procedures” with nuclear protein extracts isolated from HepG2 cell after incubation with vehicle or 100 nM TPA for 4 h. Lanes 1–11 show protein binding to the 32P-labeled wild type probe and lane 12 shows protein binding to the 32P-labeled mutated type probe. The sense strand sequence of probes are as follows: wild type probe, 5’-AAGTCCTTACTCAATAACTT, and mutated type probe, 5’-AAGTCCTTACTCGATAACTT (mutated nucleotide is underlined). Unlabeled 20-fold to 200-fold molar excess competitor DNAs were added to the binding reaction as indicated. Similar results were obtained in three independent experiments.

Fig. 3. **The TPA-induced binding protein is C/EBPβ and the TPA responsive site works as a C/EBP site.** Gel mobility shift experiments were performed as described under “Experimental Procedures” with nuclear protein extracts isolated from
HepG2 cell after incubation with vehicle (lanes 1,2), 100 nM TPA for 2 h (lanes 3,4) or for 4 h (lanes 5-14). 32P-labeled wild type probe whose sequence is shown in Fig. 2 was used in all lanes. Unlabeled 200-fold molar excess competitor DNAs were added to the binding reaction as indicated (lanes 2,4,6). Specific antibodies (1 µg) to C/EBPα (lane 8), C/EBPβ (lane 9), C/EBPδ (lane 10), c-Fos (lane 11), c-Jun (lane 12), HNF-1α (lane 13), or c-Myc (lane 14) were added to the binding reaction as described in “Experimental Procedures”. An arrow shows the supershift band which appeared only when specific antibody to C/EBPβ was added. Similar results were obtained in three independent experiments.

Fig. 4. **DNase footprinting assays confirm that the TPA inducible binding protein is C/EBPβ and the C/EBP site is protected in SK-N-MC cell nuclear extract.**

DNase footprinting assays were performed as described under “Experimental Procedures” without (lane 1) or with nuclear protein extracts isolated from HepG2 cell after incubation with vehicle (lane 2) or 100 nM TPA for 4 h (lane 3), or with nuclear protein from 293T cells transfected with control vector (pcDNA3, lane 4) or C/EBPβ expression vector (pcDNA3C/EBPβ, lane 5), or with nuclear protein from SK-N-MC cells (lane 6). Similar results were obtained in three independent experiments.

Fig. 5. **Overexpressed C/EBPβ transactivates the human IGF-I promoter-1 through the C/EBP site in 293T cells.** Wild type IGF-I promoter-1-luciferase reporter plasmid (pIGFI-300) or mutated type IGF-I promoter-1-luciferase reporter plasmid (pIGFI-300M) was cotransfected with 1 µg of control expression vector (indicated as -) or indicated amount of C/EBPβ expression vector (pcDNA3C/EBPβ) with a pMSV-β-galactosidase control vector into 293T cell using Lipofectamin. After 48 h incubation, cytoplasmic extracts were prepared, and luciferase activity was measured. Data were normalized for transfection efficiency (β-galactosidase
expression) and presented as means ± SD of at least three independent experiments performed in duplicate.

Fig. 6. **TPA stimulates C/EBPβ gene transcription in HepG2 cells.** Northern blotting analyses were performed with 10 µg of total RNA isolated from HepG2 cells after incubation with 100 nM of TPA for the times indicated. The probe was 32P-labeled rat C/EBPβ probe. Similar results were obtained in three independent experiments.

Fig. 7. **Overexpressed dominant negative type RSK inhibits C/EBPβ transactivation of IGF-I promoter-1.** **Upper panel,** IGF-I promoter-1-luciferase reporter plasmids (pIGFI-300) were cotransfected with 1 µg of control expression vector (pcDNA3, lanes 1-3) or 1 µg of C/EBPβ expression vector (pcDNA3C/EBPβ, lanes 4-6), and 1 mg of another control expression vector (pKH3, lanes 1,4) or 1 µg of HA tagged wild type RSK expression vector (WT RSK, lanes 2,5) or HA tagged dominant negative type RSK expression vector (DN RSK, lanes 3,6), and renilla luciferase (pRLCMV) control vector into 293T cell using Lipofectamin. After 48 h incubation, cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency (renilla luciferase expression) and presented as means ± SD of at least three independent experiments performed in duplicate. **Lower panel,** Western blotting with cytoplasmic extracts using anti-HA antibody was performed.

Fig. 8. **TPA-induced activation function of C/EBPβ depends on RSK.** The bar graph (a) depicts transactivation potential of GAL4·C/EBPβ chimeras. The fusion proteins were obtained by fusing the DNA-binding domain (DBD) of GAL4 (aa 1 to 147) to the 118-aa (Gal4C/EBPβ118) or 166-aa (Gal4C/EBPβ166) N-terminal region of rat C/EBPβ (b). A single aa substitution (Ser-105 to Ala) was introduced to
Gal4C/EBPβ166 plasmid to produce Gal4C/EBPβ166mut105Ala plasmid. One microgram of each GAL4-C/EBPβ fusion plasmid was cotransfected into HepG2 cells with 1 µg of Gal4-responsive reporter plasmid (pFR-Luc) and 5 ng of an internal control, pRL-CMV. Where indicated, 1µg of wild type or dominant negative type RSK expression plasmid was also cotransfected. After transfection, cells were incubated for 24 h, and then, \(10^{-7}\) M TPA or vehicle was added. After another 24-h incubation, dual-luciferase assays were performed. The firefly luciferase results were normalized with respect to transfection efficiency assessed by renilla luciferase results. The data were presented as means of at least three independent experiments performed in duplicate. AD: activation domain  RD: repression domain

**Fig. 9. Overexpressed dominant negative type RSK inhibits TPA stimulation of IGF-I promoter activity.** IGF-I promoter-1·luciferase reporter plasmids (pIGFI-300) were cotransfected with 1 mg of control expression vector or 1 mg of dominant negative type RSK expression vector as indicated and renilla luciferase (pRLCMV) control plasmid into 293T cell using Lipofectamin. After 24 h incubation, cultures were exposed to control medium (containing vehicle), or TPA (100 nM) for 24 h. Cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency assessed by renilla luciferase results and presented as means \(\pm\) SD of at least three independent experiments performed in duplicate.

**Fig. 10 PKC inhibitor decreases IGF-I mRNA in SK-N-MC cells.** Northern blotting analyses were performed with 10 µg of total RNA isolated from SK-N-MC cells after incubation with vehicle (lanes 1,2) or 100 nM of staurosporin (lanes 3,4) for 24 h. The probe was \(^{32}\)P-labeled human IGF-I cDNA probe. Similar results were obtained in three independent experiments performed in duplicate.
Fig. 11. **C/EBPβ binds to C/EBP site of IGF-I promoter-1 in SK-N-MC cells in PKC-dependent manner.** Gel mobility shift experiments were performed as described under “Experimental Procedures” with nuclear protein extracts isolated from SK-N-MC cell after incubation with vehicle (lanes 1-3, 7-14) or 100 nM TPA 4 h. 32P-labeled wild type probe whose sequence is shown in Fig.2 was used in all lanes. Unlabeled 200-fold molar excess wild type competitor (lanes 2,5) or mutated type competitor (lanes 3,6) was added to the binding. Specific antibodies (1 µg) to C/EBPα (lane 8), C/EBPβ (lane 9), C/EBPδ (lane 10), c-Fos (lane 11), c-Jun (lane 12), HNF-1α (lane 13), or c-Myc (lane 14) was added to binding reaction as described in “Experimental Procedures”. The arrow shows the supershift band which appeared only when specific antibody to C/EBPβ was added. Similar results were obtained in three independent experiments.

Fig. 12. **PKC inhibitor decreases basal promoter activity of human IGF-I promoter-1 in SK-N-MC cells.** Left panel, Diagrammatic representation of IGF-I promoter-1-luciferase reporter plasmids. The nucleotide sequence is shown for the region containing the C/EBP site (underlined). pIGFI-300M has a point mutation and the altered base is in bold. Right panel, IGF-I promoter-1-luciferase reporter plasmids were cotransfected with a renilla luciferase control plasmid into SK-N-MC cell using Lipofectamin. After 24-h incubation, cultures were exposed to control medium (containing vehicle), or GF109203X (1 µM) for 24 h. Cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency assessed by renilla luciferase results and presented as means ± SD of at least three independent experiments performed in duplicate.

Fig. 13. **Dual control of C/EBPβ activity by intrinsically activated PKC in SK-N-MC cells.** a) PKC inhibitor decreases C/EBPβ mRNA in SK-N-MC cells. Northern
blotting analyses were performed using $^{32}$P-labeled rat C/EBPβ cDNA as a probe. Ten micrograms of total RNA isolated from SK-N-MC cells after incubation with vehicle (lanes 1, 2) or 100 nM of staurosporin (lanes 3, 4) for 24 h were loaded. Similar results were obtained in three independent experiments performed in duplicate. b) Intrinsically activated PKC stimulates activation function of C/EBPβ through RSK activation. The bar graph depicts transactivation potential of GAL4·C/EBPβ chimeras. The fusion protein (Gal4C/EBPβ166) was obtained by fusing the DNA·binding domain (DBD) of GAL4 (aa 1 to 147) to the 166-aa N-terminal region of rat C/EBPβ. A single aa substitution (Ser·105 to Ala) was introduced to Gal4C/EBPβ166 plasmid to produce Gal4C/EBPβ166mut105Ala plasmid. One microgram of each GAL4·C/EBPβ fusion plasmid was cotransfected into SK-N-MC cells with 1 µg of Gal4-responsive reporter plasmid (pFR·Luc) and 5 ng of an internal control, pRL·CMV. Where indicated, 1 µg of wild type (RSK·WT) or dominant negative type RSK (RSK-DN) expression plasmid was also cotransfected. After transfection, cells were incubated in the basal condition medium for 24 h, and then, 1 µM GF109203X or vehicle was added. After another 24-h incubation, dual-luciferase assays were performed. The firefly luciferase results were normalized with respect to transfection efficiency assessed by renilla luciferase results. The data were presented as means of at least three independent experiments performed in duplicate.
Fig. 1.
Fig. 2.

| Control | TPA 4hr |
|---------|---------|
| -       | -       |
| X20 comp. | X20 comp. |
| X50 comp. | X50 comp. |
| X200 comp. | X200 comp. |
| mutated | mutated |
| mutated probe | mutated probe |

1 2 3 4 5 6 7 8 9 10 11 12
Fig. 3.
Fig. 4.

HepG2

293T

Naked  Control  TPA 4hr  -  C/EBPβ  SK-N-MC

C/EBP site

+1  +20  +40  +60

1  2  3  4  5  6
Fig. 6.

TPA(h)  0  0.5  1  2

C/EBPβ mRNA

28S

18S
Fig. 7.
Fig. 8.

a.

| Condition                                      | Relative Light Units |
|------------------------------------------------|----------------------|
| Gal4DBD, TPA(+)                                 | 2                    |
| Gal4DBD, TPA(-)                                 | 1                    |
| Gal4C/EBPβ118, TPA(+)                           | 4                    |
| Gal4C/EBPβ118, TPA(-)                           | 3                    |
| Gal4C/EBPβ166, TPA(+)                           | 6                    |
| Gal4C/EBPβ166, TPA(-)                           | 5                    |
| Gal4C/EBPβ166+RSK WT, TPA(+)                    | 8                    |
| Gal4C/EBPβ166+RSK WT, TPA(-)                    | 7                    |
| Gal4C/EBPβ166+RSK DN, TPA(+)                    | 10                   |
| Gal4C/EBPβ166+RSK DN, TPA(-)                    | 9                    |
| Gal4C/EBPβ166mut105Ala, TPA(+)                  | 12                   |
| Gal4C/EBPβ166mut105Ala, TPA(-)                  | 11                   |

b.

- Gal4C/EBPβ118
- Gal4C/EBPβ166

`AD` indicates activation domain, `RD` indicates repression domain.
Fig. 9.

- Control
- $10^{-7}$M TPA

Bar graph showing the comparison of different conditions:
- Empty plasmid
- Empty plasmid
- DN RSK
Fig. 10.
Fig. 11.

| Cont. | Stauro. |
|-------|---------|
| X200 comp. | X200 mutated |
| - | - |

- anti-C/EBPα
- anti-C/EBPβ
- anti-C/EBPδ
- anti-c-Fos
- anti-c-Jun
- anti-HNF-1α
- anti-c-Myc

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fig. 12.

![Diagram showing the relationship between nucleotides and relative light units for different constructs: pA3Luc, pIGFI-1600, pIGFI-500, pIGFI-300, and pIGFI-300M. The diagram includes promoter and exon elements with nucleotide coordinates and a y-axis for relative light units.]
Fig. 13a.

Control  Stauro.

C/EBPβ mRNA

28S

18S

1  2  3  4
Fig. 13b.
Protein kinase C-dependent, CCAAT/enhancer binding protein β-mediated expression of insulin-like growth factor I gene

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