Effects of Thalidomide on Bone Sialoprotein Gene Transcription

Kyung Mi Kim,¹ Zhitao Wang,^{1,3} Ayako Kato,¹ and Yorimasa Ogata^{1,2}

¹Departments of Periodontology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan.

²Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan.

³Tianjin Stomatology Hospital, Tianjin, China.

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Abstract

Thalidomide is an immunomodulatory drug and primarily prescribed as a sedative and hypnotic. Treatment with thalidomide inhibited osteoblast development. Bone sialoprotein (BSP) is a mineralized tissue-specific protein that appears to function in the initial mineralization of bone. BSP expression is essentially restricted to differentiated cells of mineralized connective tissues and that the expression of BSP is developmentally regulated. To determine molecular mechanisms involved in the suppression of bone formation we have analyzed the effects of thalidomide on BSP gene expression. Thalidomide (10 μ g/ml) decreased BSP mRNA levels at 12 h in ROS 17/2.8 osteoblast-like cells. Transient transfection analyses, using chimeric constructs of the rat BSP gene promoter linked to a luciferase reporter gene, revealed that thalidomide (10 μ g/ml, 12 h) suppressed luciferase activities of pLUC3 (nts -116 to +60), pLUC4 (nts -425 to +60) and pLUC5 (nts -801 to +60) transfected into ROS17/2.8 cells. Further deletion analysis of the BSP promoter showed that a region within nts -43 to -84 was targeted by thalidomide. Introduction of 2 bp mutations in the inverted CCAAT box (ATTGG; nts -50 and -46), a putative cAMP response element (CRE; nts -75 to -68), a fibroblast growth factor 2 (FGF2) response element (FRE; nts -92 to -85) and a pituitary-specific transcription factor-1 (Pit-1) binding site (Pit-1; nts -111 to -105) showed that the thalidomide effects were mediated by the CRE and inverted CCAAT box. Gel mobility shift analyses showed that thalidomide increased nuclear protein binding to CRE. Whereas, inverted CCAAT box binding proteins did not change after stimulation by thalidomide. Antibodies against CRE-binding protein 1 (CREB1), phospho-CREB1, c-Fos, c-Jun, JunD and CREB2 disrupted CRE-protein complex formation. These studies, therefore, demonstrated that thalidomide suppresses BSP gene transcription mediated by CRE and inverted CCAAT box in the rat BSP gene promoter.

Keywords :

Thalidomide, bone sialoprotein, osteoblast, transcription

Introduction

Thalidomide was sold as a sedative and was prescribed to pregnant woman as a treatment for morning sickness. Use of thalidomide during weeks 3 to 8 of gestation causes multiple birth defects such as limb, ear, cardiac and gastrointestinal malformations. Thalidomide is potent immunomodulators with biologic effects on both cytokine stimulation and cell-mediated immunity (1, 2). Thalidomide enhances degradation of tumor necrosis factor- α (TNF- α)

mRNA, thus suppressing this proinflammatory cytokine release from endotoxin-stimulated monocytes and macrophages (3). Previous studies have suggested thalidomide-induced oxidative stress and its antiangiogenic action as a possible cause of teratogenicity (4, 5). Recently, thalidomide is used in the treatment of leprosy and multiple myeloma, although it causes teratogenicity. If thalidomide is used under strict control and removal of its side effects is desirable for wide applications of this potentially useful drug (6). Cereblon (CRBN) was identified as a thalidomide-binding protein, which forms an E3 ubiquitin ligase complex with damaged DNA binding protein 1 (DDB1) and Cul4A

Correspondence to :

Yorimasa Ogata

E-mail : ogata.yorimasa@nihon-u.ac.jp

that is important for limb out growth and expression of the fibroblast growth factor 8 (FGF8) in zebrafish and chicks. Thalidomide initiates its teratogenic effects by binding to CRBN and inhibiting the associated ubiquitin ligase activity(7). Treatment with thalidomide and thalidomide derivatives with immunomodulatory functions such as lenalidomide or pomalidomide significantly inhibited osteoblast development *in vitro*, as reflected by a reduction of alkaline phosphatase activity and matrix mineralization(8).

Bone sialoprotein (BSP) is a sulfated and phosphorylated glycoprotein found almost exclusively in mineralized tissues(9, 10). The temporo-spatial deposition of BSP into the extracellular matrix of bone(11, 12) and the ability of BSP to nucleate hydroxyapatite crystal formation(13) indicate a potential role for BSP in the initial mineralization of bone. BSP is also expressed in several pathologies in which mineralization occurs including malignant breast cancer where it is associated with the formation of ectopic hydroxyapatite microcrystals(14-16). Thus, regulation of BSP gene transcription appears to be important in the differentiation of osteoblasts, for bone matrix mineralization and for pathologic mineralization. To study the transcriptional regulation of BSP gene expression, we and other groups have cloned and sequenced the rat, human and mouse BSP gene promoters(17-19), which are highly conserved within a 370 bp proximal region. This region includes an inverted TATA box(20) overlapping a vitamin D response element(21) and an inverted CCAAT box (-50 to -46), which is required for basal transcriptional activity(22, 23). In addition, a fibroblast growth factor 2 (FGF2) response element (FRE; -92 to -85)(24), a pituitary-specific transcription factor-1 (Pit-1) motif (-111 to -105), that mediates the stimulatory effects of parathyroid hormone(25), and a homeodomain protein-binding site (HOX; -199 to -192)(26), have been characterized. Further upstream in the rat promoter a transforming growth factor- β activation element (-499 to -485)(27) and a glucocorticoid response element (-920 to -906), overlapping an AP1 site (-921 to -915)(10, 28) has also been identified.

To elucidate molecular pathways through which thalidomide suppresses bone formation we have analyzed the effects of thalidomide on BSP gene expression in ROS 17/2.8 cells. These studies show that thalidomide exerts suppresses transcription of the BSP gene through an inverted CCAAT and a CRE in the rat BSP gene promoter.

Materials and Methods

Materials

Cell culture media, fetal calf serum (FCS), Lipofectamine, penicillin and streptomycin, trypsin were obtained from Invitrogen (Carlsbad, CA, USA). The pGL3-promoter vector and pSV- β -Galactosidase (β -Gal) control vector were purchased from Promega (Madison, WI, USA). Thalidomide was from Sigma (St. Louis, USA). All chemicals used were of an analytical grade.

Cell culture

The rat clonal cell line, ROS 17/2.8 (generously provided by Dr. Jaro Sodek) was used in these studies as an osteoblastic cell line that synthesizes BSP(10, 29). Cells grown to confluence in 60 mm tissue culture dishes in α -MEM medium containing 10% FCS were changed to α -MEM without serum and incubated with or without 10 μ g/ml thalidomide for time periods extending over 3-24 h to determine the effects of thalidomide on the expression of BSP mRNA. RNA was isolated from triplicate cultures at various time intervals and analyzed for the expression of BSP mRNA by Northern hybridization as described below.

Northern hybridization

Total RNA from the ROS 17/2.8 was extracted with guanidium thiocyanate and, following purification, 20 μ g aliquots of RNA were fractionated on 1.2% agarose gel and transferred onto a Hybond XL membrane, as described previously(27). Hybridizations were performed at 42°C with a 32 P-labeled rat BSP, a rat osteopontin (OPN) or a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Following hybridization, membranes were washed four times for 5 min each at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate pH 7.0 containing 0.1% SDS. This was followed by two, 20 min washes at 55°C in 15 mM sodium chloride, 1.5 mM trisodium citrate pH 7.0, 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6-kb and 2.0-kb) of rat BSP mRNA, were scanned in a Bio-imaging analyzer (BAS 2000, Fuji BAS 2000).

Transient transfection assays

Exponentially growing ROS 17/2.8 cells were used for the transfection assays. Twenty-four hours after plating, cells at 50-70% confluence were transfected using a Lipofectamine reagent. The transfection mixture included 1

μg of a luciferase (LUC) construct and $2\mu\text{g}$ β -Gal control vector as an internal control (10). One day post-transfection, cells were deprived of serum for 12 h and $10\mu\text{g}/\text{ml}$ thalidomide was added for 12 h prior to harvesting. The luciferase assay was performed according to the supplier's protocol (PicaGene, Toyo Inki, Tokyo, Japan) using a Luminescence reader (Acuu Flex Lumi 400; Aloka, Tokyo, Japan) to measure the luciferase activities. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce the dinucleotide substitutions using the Quickchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). All constructs were sequenced as described previously to verify the fidelity of the mutagenesis.

Gel mobility shift assays

Confluent ROS 17/2.8 cells in four T-75 flasks incubated for 3, 6 and 12 h with $10\mu\text{g}/\text{ml}$ thalidomide in α -MEM without serum were used to prepare nuclear extracts. Nuclear protein was extracted by the method of Dignam et al. (30) with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, $2\mu\text{g}/\text{ml}$ leupeptin, $2\mu\text{g}/\text{ml}$ pepstatin A, $1\mu\text{g}/\text{ml}$ aprotinin, pH 7.9). Double-stranded oligonucleotides encompassing the inverted CCAAT (nts -61 to -37, 5'-CCGTGACCGTGATTGGCTGCTGAGA), the cAMP response element (CRE; nts -84 to -59, 5'-CCCACAGCCTGACGTCGCACCGGCCG), the FRE (nts -98 to -79, 5'-TTTTCTGGTGAGAACCCACA), and Pit-1 (nts -115 to -96, 5'-CGTGTTGTAGTTACG GATTT) were prepared. For gel shift analysis the double-stranded-oligonucleotides were end-labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase. Nuclear protein extracts ($3\mu\text{g}$) were incubated for 20 min at room temperature (21°C) with 0.1 pM radiolabeled double-stranded oligonucleotide in buffer containing 60 mM NaCl, 5 mM MgCl_2 , 0.1 mM EDTA, 15 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 5% glycerol and $0.1\mu\text{g}/\text{ml}$ poly (dI-dC). For competition experiments unlabelled oligonucleotides for the CRE (TGACGTCG) and mutation CRE (mCRE; cGACGcCG) were used at 40-fold molar excess. Following incubation, the protein-DNA complexes were resolved by electrophoresis on 5% non-denaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 150 V at RT. Following electrophoresis, the gels were dried and autoradiograms prepared and analyzed using an image analyzer. Supershift experiments

were performed using anti-CRE-binding protein 1 (CREB1, p43; Rockland), phospho-CREB1 (Ser133; Upstate), c-Fos (sc-253), c-Jun (sc-44), Fra-2 (sc-604), JunD (sc-74) and CREB2 (sc-200) (Santa Cruz Biotechnology) antibodies. Antibodies were added to each reaction mixture and incubated for 4 h at 4°C before electrophoresis was performed under the same conditions as described above.

Statistical analysis

Triplicate samples were analyzed for each experiment, which was replicated at least three times to ensure consistency of the responses to thalidomide. Significant differences between control and treatment were determined using the oneway ANOVA.

Results

Suppression of BSP mRNA Expression in ROS 17/2.8 Cells

We studied the effect of thalidomide on BSP mRNA expression in osteoblastic ROS 17/2.8 cells. To determine the dose-response for thalidomide regulation of BSP, confluent ROS 17/2.8 cells were treated with different concentrations of thalidomide for 12 h. BSP mRNA levels were determined by Northern blot analysis. Thalidomide decreased BSP mRNA expression maximal at $10\mu\text{g}/\text{ml}$ (Fig. 1A). Thus, $10\mu\text{g}/\text{ml}$ of thalidomide was used to determine a time course of BSP mRNA expression (Fig. 1B). Thalidomide suppressed BSP mRNA levels at 12 h. Osteopontin mRNA levels were decreased slightly at 3, 6 and 12 h (Fig. 1B), whereas no effect on GAPDH mRNA was observed (Fig. 1A and B).

Transient Transfection Analysis of Rat BSP Promoter Constructs

To determine how thalidomide regulates BSP transcription via sequences from the 5'-flanking region of the BSP gene were tested for transcriptional activity and responsiveness to thalidomide by transient transfection of chimeric constructs in ROS 17/2.8 cells. The constructs used (pLUC1~pLUC5) and their responsiveness to thalidomide are shown in Fig. 2. The transcriptional activity of pLUC3, which encompasses nucleotides -116 to +60, was reduced and almost the same levels of decrease were observed in constructs pLUC4 and pLUC5. However, deletion of the sequence between -116 and -43 abolished the thalidomide-mediated reduction (pLUC2 and pLUC1). Within the DNA sequence of pLUC3 that is unique in this

Fig.1 Northern hybridization analysis of thalidomide effects on BSP mRNA expression. **A:** Dose-response effect (0.001, 0.1 and 10 $\mu\text{g/ml}$) of thalidomide on BSP mRNA levels in ROS17/2.8 osteoblast-like cells treated for 12 h. **B:** A 12 h time-course revealed a decrease in BSP mRNA at 12h after the administration of 10 $\mu\text{g/ml}$ thalidomide to ROS17/2.8 cells. Total RNA was isolated from triplicate cultures harvested after incubation times of 3, 6 and 12 h, and used for Northern hybridization analysis using a ^{32}P -labelled rat BSP DNA probe and a GAPDH DNA probe. The hybridized bands, representing the two polyadenylated forms (1.6-kb and 2.0-kb) of rat BSP mRNA, were scanned in a Bio-imaging analyzer.

region (nts -116 and -43), is an inverted CCAAT box (ATTGG; nts -50 and -46), a cAMP response element (CRE; nts -75 to -68), a fibroblast growth factor 2 response element (FRE; nts -92 and -85), a putative NF κ B site (nts -93 and -102) and a pituitary-specific transcription factor-1 (Pit-1) motif (between nts -111 and -105), which is a target of parathyroid hormone stimulation (Fig.3). To more closely define the regulatory element between nts -116 and -43 that is utilized by thalidomide,

we prepared a series of 5' deletion constructs. -60BSPLUC, -84BSPLUC and -108BSPLUC constructs suppressed by thalidomide (Fig. 4). The results showed that the element responding to thalidomide was present between nts -84 and the transcription start site in the BSP promoter (Fig. 3 and 4). Next, we introduced 2-base pair mutations in the pLUC3 construct. Whereas mutations in the FRE and Pit-1 had no effect on thalidomide, mutations of the CCAAT and CRE significantly reduced the thalidomide effects on the transcriptional activity (Fig. 5).

Gel Mobility Shift Assays

To clarify nuclear proteins that bind to the inverted CCAAT and CRE and mediate the thalidomide effects on transcription, double-stranded oligonucleotides were end-labeled and incubated with equal amount (3 μg) of nuclear proteins extracted from confluent ROS 17/2.8 cells that were either not treated (control) or treated with 10 $\mu\text{g/ml}$ thalidomide for 3, 6 and 12 h. Component of the sequence-specific heterotrimeric nuclear factor Y (NF-Y) transcription factor which specifically recognizes a CCAAT motif found in the promoters of its target genes. When we used the inverted CCAAT sequence as a probe, the DNA-NF-Y protein complex(22, 23) did not change after thalidomide stimulation (Fig. 6, lanes 1~4). After thalidomide stimulation for 3 h, CRE-protein complex formation was increased, decreased at 6 h and increased again at 12 h (Fig. 6, lanes 5~8). On the other hand, FRE- and Pit-1-protein complexes formations did not change (Fig. 6, lanes 9~16). That the DNA-protein complex represents a specific interaction was indicated by competition experiments in which 40-fold excess of CRE reduced the amount of complex formed (Fig. 7, lane 3). In contrast, 40-fold excess mCRE did not compete with complex formation (Fig. 7, lane 4). CRE-binding protein was supershifted by phospho-CREB1 antibody (Fig. 7, lane 7), and disrupted by CREB1, c-Fos, c-Jun, JunD and CREB2 antibodies (Fig. 7, lanes 6, 8, 9, 11, 12)

Discussion

In this study we show that thalidomide suppresses the expression of BSP, a major protein of the mineralized connective tissues which is involved in osteogenic differentiation and the mineral formation. Treatment of ROS 17/2.8 cells with thalidomide decreased the BSP mRNA levels (Fig.1), reflecting a reduction of gene transcription measured by transient transfection assays. The results of

Fig.2 Thalidomide down-regulates BSP promoter activity. Transient transfections of ROS 17/2.8 cells, in the presence or absence of thalidomide (10 μ g/ml) for 12 h, were used to determine transcriptional activity of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase reporter gene. The results of transcriptional activity obtained from three separate transfections with constructs; pLUC basic (pLUCB) and pLUC1 to pLUC5 have been combined and the values expressed with standard errors. Significant differences from control: ** ($P < 0.01$).

Fig.3 A: Regulatory elements in the proximal rat BSP promoter. The inverted TATA and CCAAT boxes, cAMP response element (CRE), FGF2 response element (FRE), pituitary-specific transcription factor-1 (Pit-1) motif, homeodomain protein-binding site (HOX), TGF- β activation element (TAE) overlapping with activator protein 2 (AP2) and glucocorticoid response element (GRE) overlapping the API, a vitamin D response element (VDRE) that overlaps the inverted TATA box are present within a proximal promoter are shown. B: The nucleotide sequences of proximal promoter region of the rat BSP gene are shown from nucleotides -201 to -35. The inverted CCAAT box, CRE, Runx2, FRE, nuclear factor-kappa B (NF κ B), Pit-1, AP1, another Runx2 and HOX are present.

Fig.4 Fine 5'-deletion mapping of the nts -116 to -43 in the BSP promoter. A series of rat BSP promoter 5' deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for reduction in the presence of thalidomide (10 μ g/ml) for 12 h. The results demonstrated that the thalidomide response region exists in between -84 and -43 in the BSP promoter. The results of transcriptional activity obtained from three separate transfections with constructs; -43BSPLUC (-43 to +60), -60BSPLUC (-60 to +60), -84BSPLUC (-84 to +60), -108 BSPLUC (-108 to +60), -116 BSPLUC (-116 to +60) have been combined and the values expressed with standard errors. Significant differences from control: **($P < 0.01$), *($P < 0.05$).

Fig.5 CRE and inverted CCAAT in the rat BSP promoter are necessary for suppressive effect of thalidomide. Dinucleotide substitutions were made within context of the homologous -116 to +60 BSP promoter fragment (pLUC3). M-CCAAT (ATTt), M-CRE (cGACGcCG), M-FRE (GGcaAGAA) and M-Pit1 (TTacAGT) constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined the effects of thalidomide for 12 h. The results of transcriptional activities obtained from three separate transfections with constructs; pLUCB, pLUC3, M-CCAAT, M-CRE, M-FRE and M-PIT were combined and the values expressed with standard errors. Significant differences from relative luciferase activity of pLUC3: **($P < 0.01$), *($P < 0.05$).

Fig.6 Gel mobility shift assays using inverted CCAAT, CRE, FRE and Pit-1. After stimulation by 10 μ g/ml thalidomide (3, 6 and 12 h), CRE-protein complexes were increased at 3 h, decreased at 6 h and increased again at 12 h (lanes 5-8). CCAAT-, FRE- and Pit-1-protein complexes did not change after stimulation by thalidomide (lanes 1-4, 9-12, 13-16). DNA-protein complexes were separated in a 5% polyacrylamide gel with a low-ionic-strength Tris-borate buffer, dried under a vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

transient transfection assays showed thalidomide response region was nucleotide sequence between -84 to -43 from the transcriptional start site (Fig.2 and 4). From the mutation luciferase assays the sites of transcriptional regulation by thalidomide could be identified as inverted CCAAT box and CRE in the proximal promoter of the BSP gene (Fig. 5).

Basal transcription of the BSP and OPN genes is mediated by an inverted CCAAT box that can be stimulated by v-Src(22, 31). The major transcription factor that binds the inverted CCAAT box is NF-Y, which is essential for the expression of the class II genes of the major histocompatibility complex (MHC), and is involved in the regulation of albumin and type I collagen genes(32, 33). Also, activation of BSP gene transcription by flavonoids is mediated through an inverted CCAAT box in ROS17/2.8 cells(23). Notably, the CCAAT sequence is conserved in the rat, mouse and human BSP gene promoters(17-19), and is also present at a similar location in the OPN gene promoter(31), indicative of a potentially important role in transcriptional regulation of the BSP and the OPN genes. Inverted CCAAT box is bound

with high affinity by an ubiquitous trimeric complex, NF-Y transcription factor. This complex is composed of three conserved subunits, NF-YA, NF-YB and NF-YC(32, 34), all of which are required for DNA binding. NF-Y is also required for cyclin B1 expression in terminally differentiated skeletal muscle cells depends upon the loss of a functional NF-Y complex(35). That the promoters of several cell cycle regulatory genes such as cyclin A, cyclin B1, cyclin B2, cdk1 and cdc25C contain CCAAT boxes suggests NF-Y is a key regulator of cell cycle(35). However, the regulatory mechanism of NF-Y mediated gene transcription is not well understood. In the gel shift assays, the CCAAT-NF-Y complex did not change after stimulation with thalidomide (Fig.6). Therefore, it is likely that thalidomide treatment results in modification of NF-Y that influence its transcriptional properties, but these changes do not affect the binding of the NF-Y protein complex to the CCAAT box.

That the CRE mediates the effects of thalidomide on BSP was initially determined by transient transfection assays in which promoter constructs spanning the proximal promoter

Fig. 7 Specific binding of nuclear proteins to CRE. Radiolabeled double-stranded CRE was incubated with nuclear protein extracts (3 μ g) obtained from ROS 17/2.8 cells incubated without (Control; lane 1) or with 10 μ g/ml thalidomide for 3 h (lanes 2–12). Competition reactions were performed using a 40-fold molar excess of unlabeled CRE (CCCACAAGCCTGACGTCGCACCGGCCG; lane 3) and mutation-CRE (mCRE; CCCACAAGCCcGACGcCGCACCGGCCG; lane 4) oligonucleotides. Supershift experiments were performed with 0.4 μ g of preimmune IgG (lane 5) and antibodies against CREB1 (lane 6), phospho-CREB1 (lane 7), c-Fos (lane 8), c-Jun (lane 9), Fra2 (lane 10), JunD (lane 11) and CREB2 (lane 12) were added separately to each reaction. DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under a vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

(–84 and –43; Fig. 4) of the BSP gene were used. This region encompasses an inverted CCAAT box (nts –50 and –46), a CRE (nts –75 and –68) and a putative Runx2 (nts –84 and –79) (Fig. 3). Mutation of the inverted CCAAT and CRE was used to demonstrate that the effects of thalidomide could be blocked in the absence of CCAAT and CRE (Fig. 5). Thus, to further establish the site of BSP gene regulation, gel mobility shifts were used to identify proteins in nuclear extracts of ROS 17/2.8 cells that selectively bound to the CRE and inverted CCAAT sequences (Fig. 6). CRE-binding proteins were increased at 3 h, decreased at 6 h and increased again at 12 h (Fig. 6, lanes 5–8). In contrast, the protein binding to the inverted CCAAT did not change after stimulation with thalidomide as previously described.

CRE was first identified as an inducible enhancer of genes that can be transcribed in response to an increased cAMP level. Multiple CRE-binding proteins such as CREB(36), ATFs(37) and C/EBPs(38) have been identified. All of these proteins have a basic region adjacent to a leucine

zipper motif (bZip domain)(39). The leucine zipper is responsible for the dimerization of the protein and dimerization is a prerequisite for DNA binding(40). The CRE is bound by CREB homodimers, ATF2 homodimers, ATF3 homodimers, C/EBP family members and Jun-Fos family heterodimers(41). A consensus CRE is constituted by an 8 bp palindromic sequence (TGACGTCA) (42). The CRE in BSP promoter is identical in sequence (TGACGTCG) to the consensus CRE except for a single, A to G, nucleotide change(25). Results of supershift assay showed that CRE binding protein complexes were phospho-CREB1, c-Fos, c-Jun, JunD and CREB2 (also designated ATF4) (Fig. 7). Although CRE binding protein complexes increased by thalidomide at 12 h, BSP transcription decreased by thalidomide treatment at 12 h. Further study necessary to elucidate the mechanism of opposite directed BSP gene transcription by thalidomide.

In this study, therefore, we have shown that thalidomide suppresses BSP expression via inverted CCAAT and CRE elements, which is conserved in mammalian promoters of

BSP, has been identified as the target of thalidomide-mediated regulation of BSP gene transcription.

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