Blockade of AP1 Transactivation Abrogates the Abnormal Expression of Breast Cancer-specific Gene 1 in Breast Cancer Cells*

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Breast cancer-specific gene 1 (BCSG1) is not expressed in normal breast tissue but is highly expressed in the vast majority of invasive and metastatic breast carcinomas. When over-expressed, BCSG1 significantly stimulates the proliferation and invasion of breast cancer cells. The accumulated evidence suggests that the aberrant expression of BCSG1 in breast carcinomas is caused by transcriptional activation of the BCSG1 gene. However, the transcription factors that activate BCSG1 transcription have not been identified. In this study, we extensively investigated the role of AP1 in BCSG1 expression in breast cancer cells. We demonstrate that there are two closely located AP1 binding sites residing in the first intron of the BCSG1 gene. Mutation of either AP1 motif on the BCSG1 promoter constructs markedly reduces the promoter activity. We further show that 12-O-tetradecanoylphorbol-13-acetate (TPA) increases BCSG1 mRNA expression and up-regulates BCSG1 promoter activity through the intronic AP1 sites. The effect of TPA on BCSG1 transcription is also demonstrated under in vivo conditions in intact cells by using chromatin immunoprecipitation assays that show the TPA-induced binding of c-Jun to the chromatin region encompassing the intronic AP1 sites. Finally, to examine the direct effect of AP1 transactivation on BCSG1 expression, we established stable cell lines of T47D that express the dominant negative mutant of c-Jun, TAM67. RT-PCR and Western blot analyses demonstrated that levels of BCSG1 mRNA and protein in TAM67 transfectants were drastically reduced as compared with mock-transfected cells. Furthermore, inhibition of BCSG1 expression by blocking AP1 transactivation produced a similar repressive effect on cell growth as that by expressing BCSG1 antisense mRNA. We show that the anchorage-independent growth of T47D cells expressing either TAM67 or BCSG1 antisense mRNA is significantly inhibited. Taken together, we provide strong evidence that AP1 plays an overriding role in the transcription of the BCSG1 gene and that blockade of AP1 transactivation down-regulates BCSG1 expression and suppresses tumor phenotype.

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‡ The abbreviations used are: BCSG1, breast cancer-specific gene 1; AP1, activator protein 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoresis mobility shift assay; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OM, oncostatin M; TPA, 12-O-tetradecanoylphorbol-13-acetate; RT, reverse transcription; RSV-β-gal, Rous sarcoma virus-β-galactosidase.
assays showed that the luciferase reporter construct containing the 5′-flanking region and the exon 1 of the BCSG1 gene produced a low level of luciferase activity; this basal activity is mediated primarily through a GC-rich region located immediately upstream of the transcription initiation sites. However, the promoter reporter activity was markedly increased by inclusion of the intron 1 region of the BCSG1 gene. Further deletion analysis localized a consensus AP1 binding site (TGACTCTCA) in the intron that was largely responsible for the increased promoter activity (7). These previous studies suggest that the activator protein AP1 may regulate BCSG1 transcription, possibly through the intronic AP1 binding site.

Because BCSG1 expression stimulates breast tumor disease progression and AP1 has been demonstrated to play an important role in tumorigenesis (8–10), activation of BCSG1 transcription might be one of the molecular mechanisms responsible for the promoting role of AP1 in breast cancer disease development and progression. Therefore, in this report, we extensively investigated the functional role of AP1 in BCSG1 transcription. Our studies clearly demonstrate that BCSG1 is a new target gene of AP1 and that AP1 plays an overriding role in BCSG1 transcription in breast cancer cells.

MATERIALS AND METHODS

Cells and Reagents—Human breast cancer cell lines SKBR-3 and T47D were obtained from American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO). Human hepatoma cell line HepG2 was cultured in Eagle’s minimum essential medium supplemented with 10% FBS. 12-O-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma. Antibodies directed to BCSG1 (E-20), c-Jun (sc-44), c-Fos (sc-82), and β-actin were obtained from Santa Cruz Biotechnology. The plasmid pcDNA3.1-TAM67, encoding a c-Jun mutant, TAM67, was generously provided by Dr. Michael J. Birrer, National Institutes of Health (Rockville, MD).

Transient Transfection Assays—SKBR-3 and T47D cells cultured in 24-well plates at a density of 0.12 × 10^6 cells/well were transfected with a total of 200 ng of reporter DNA and 2 ng of pRL-SV40 (Renilla, Promega) well mixed with Effectene reagent (Qiagen, Valencia, CA). 40 h after transfection, harvested cells were used for Phopshoimunoprecipitation (11). The BCSG1 promoter luciferase reporter constructs were cotransfected with RSV-β-galactosidase vector (pCMV-β-lacZ) to normalize the transfection efficiency. For every cell line, triplicate wells were assayed for transfection efficiency. For every cell line, triplicate wells were transfected with the same amount of reporter DNA and the endogenous plasmid pcDNA3.1 vector (pcDNA3.1-TAM67) and the empty vector (pcDNA3.1) were used as negative controls. 40 h after transfection, luciferase activities were measured (11). The transfected cells were cultured in medium containing 0.5% FBS for 12–14 days, individual clones resistant to G418 at a concentration of 500 μg/ml were isolated and propagated in individual stable cell lines. Western blotting using anti-c-Jun antibody confirmed that the 29 kDa albumin, and 4 μg of nuclear extract in a final volume of 20 μl. Nuclear extracts were incubated with 1 ng of 32P-labeled probe (40–80 × 10^3 cpm) for 10 min at room temperature. The reaction mixtures were loaded onto a 6% polyacrylamide gel and run in TGE buffer (50 mM Tris base, 400 mM glycine, 1.5 mM EDTA, pH 8.5) at 180 V for 2–3 h at 4 °C. The gel was dried and visualized by autoradiography. In competition analysis, nuclear extracts were incubated with a 100-fold molar excess of unlabeled competitor DNA for 5 min prior to the addition of the labeled probe. For supershift assays, antibody was incubated with nuclear extract for 30–60 min at room temperature prior to the addition of the probe.

Dense sequence analysis of the EMSA probes was as follows: BCSG1-AP1, 5′-GGGAGCTCAACATGATGCAGCTGTTG-3′; AP1-MU1, 5′-GGGGATCTACCCCACTGATCAGCTGTTG-3′; AP1-MU2, 5′-GGGAGCTCAACATGATGCAGCTGTTG-3′; Sp1, 5′-TTGACCCATCCTTCCCTAGTTAG-3′. The mutated sequences are italic and underlined.

Site-directed Mutagenesis—Mutant BCSG1 promoter reporters BCSG1967AP1-MU1 and AP1-MU2 were generated by site-directed mutagenesis on the template DNA (BCSG1967) with the QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA). The correct mutation at the consensus AP1 site (TGACTCTCA→TACTCA) in the vector BCSG1967AP1-MU2 and at the AP1-like sequence on the reverse strand (GAGTCTC→GAAATCA) in BCSG1967AP1-MU1 were verified by dideoxy sequencing.

Chromatin Immunoprecipitation (ChIP) Assays with Antibody to c-Jun—Control or TPA 24-h-treated cells were cross-linked with 1.5% formaldehyde for 5 min, then was diluted to a total volume of 100 μl with 0.1 mg/ml of proteinase K at 45 °C for 1 h and purified by repeated phenol/chloroform extraction and ethanol precipitation. The purified DNA (designated as bound) was dissolved in 20 μl of Tris buffer (10 mM Tris, pH 8.5). The DNA isolated using the same procedure with omission of the step of immunoprecipitation was designated as the input DNA and was diluted 100-fold prior to be used in the PCR reaction. The bound and input DNA were analyzed by PCR (30 cycles) with primers (AP1CHIP-5′, AP1CHIP-3′) that amplify BCSG1 intron 1 region (+549 to +748). The PCR conditions were 94 °C for 5 min, 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The 200-bp PCR product was visualized on 2% agarose gel and stained with ethidium bromide. To examine the mutational effect on intronic AP1 sites on endogenous c-Jun binding, SKBR-3 cells were transiently transfected with BCSG1 promoter luciferase construct BCSG1967AP1-MU2. The transfected cells were untreated or treated with TPA for 24 h. The ChiP assays were conducted as described above with two sets of primers that amplify the open reading frame and the endogenous BCSG1 gene separately. For this purpose, the plasmid pcDNA3.1-TAM67, encoding a c-Jun mutant, TAM67, was transfected into T47D cells by the BioRad Fluoro-S MultiImager System and quantified by the program Quantity One. The PCR products were scanned using a Bio-Rad Bio-Safe DNA Concentration System and quantified by the program Quantity One. Different amounts of template DNA were tested in PCR reaction to ensure a linear range of DNA amplification.

The following primers were used in the ChiP assays: for endogenous AP1 sites, AP1CHIP-5′ (5′-agtgttctcctccgctcctc-3′) and AP1CHIP-3′ (5′-ggcagcagacgttgctcctc-3′); for the mutated AP1 site on plasmid DNA, AP1CHIP-5′ (5′-agtgttctccttccgctcctc-3′) and AP1CHIP-3′ (5′-gcttcacgactggcctaggc-3′).

Stable Transfection of T47D Cells with TAM67—TAM67 expression vector (pcDNA3.1-TAM67) and the empty vector (pcDNA3.1) were transfected separately into T47D cells using Effectene reagent. After 10–14 days, individual clones resistant to G418 at a concentration of 500 μg/ml were isolated and propagated into individual stable cell lines. Western blotting using anti-c-Jun antibody confirmed that the 29 kDa TAM67 was expressed only in the T47D-TAM67 cells and not in the vector-transfected cells.

Stable Expression of BCSG1 Antisense mRNA in T47D Cells—A 285-bp DNA fragment corresponding to the exon 1 region (−169 to +116 of BCSG1 gene) was amplified from the plasmid pBS-BCSG17159 (7) and subcloned into the EcoRI site of the expression vector pcdNA3.1(−). The antisense or sense orientation of exon 1 in the pcdNA3.1 vector was determined by restriction enzyme digestion and was verified by DNA sequencing. Vectors expressing BCSG1 antisense
mRNA (pcDNA-BCSG1-Aa) or BCSG1 sense mRNA (pcDNA-BCSG1-S) was separately transfected into T47D cells using Effectene reagent. Stable cell lines were established using the same procedures as described for TAM67 cell lines. The expression of BCSG1 antisense and sense mRNAs (285 bp) was confirmed by RT-PCR reaction. For antisense mRNA, the primer sets are: T7 as the forward primer, 5'-TATACTGACTCATAAGG-3', and BCSG-W as the reverse primer, 5'-ACGCCAGGGCTGGTGCTC-3'. The primer sets for detection of sense mRNA are: T7 as the forward primer, 5'-TAACTACGACTCATAAGG-3' and BCSG-Wr as the reverse primer, 5'-CCTGCTTGGTCTTTCACC-3'.

Western Blot Analysis—Total cell lysates were isolated from cells as described previously (34). Fifty µg of protein of total cell lysate per sample was separated on 15% SDS-PAGE, transferred to nitrocellulose membranes, blotted with rabbit anti-BCSG1 polyclonal antibody (1: 2000 dilution) or rabbit anti-c-Jun polyclonal antibody using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences). Membranes were stripped and rebotted with anti-ß-actin monoclonal antibody to normalize the amount of protein loaded on gels.

Soft Agar Colony Assays—Anchor-age-independent growth was carried out in 24-well culture plates. The bottom layer consisted of 375 µl of 5% FBS/RPMI containing 0.5% agar. The top layer consisted of 375 µl of 5% FBS/RPMI containing 0.33% agar and 2 × 10⁴ cells. The cells were cultured in an atmosphere of 5% CO₂/95% air under saturating concentrations of humidity at 37°C. After 7 days, the number of colonies was counted under a Nikon microscope at 200× amplification. Eight fields were randomly selected in each well using a Whipple glass ring with a 10 × 10 grid, and colonies of >10 cells and 6–10 cells were counted separately.

RESULTS

Our previous study identified a consensus AP1 binding site (TGACTCTA) at the region +612 to +618, relative to the translation start codon, within the first intron of the BCSG1 gene (7). An in-depth sequence analysis identified a second AP1-like sequence (TGACCTCA) at positions +605 to +598 on the reverse strand of the intron 1. These two AP1 motifs are separated by only six nucleotides. To determine whether these two AP1 sequences are both functionally involved in BCSG1 transcription, mutations were introduced individually on each site to block AP1 binding. The BCSG1 luciferase reporter constructs carrying the wild-type and the mutated AP1 sites are depicted in Fig. 1. These reporters were transiently transfected into SKBR-3, T47D, and HepG2 cells. As shown in Fig. 2, the promoter activity of BCSG1967 was 5–7-fold higher than that of BCSG1759 and BCSG1864, which do not contain the intronic AP1 sites. Mutation at either AP1 site markedly reduced the promoter activity of BCSG1967 in all three cell lines. It appeared that disruption of the consensus AP1 site (AP1-MU2) produced a stronger inhibitory effect on BCSG1 transcription than the mutation at the AP1 homologous sequence (AP1-MU1). To confirm that the decreased promoter activities of the mutant vectors were caused by disruption of the binding of AP1 to the intronic AP1 sites, we conducted a competition binding assay using an [32P]-labeled oligonucleotide probe containing the AP1 motifs and nuclear extract of SKBR-3 cells (Fig. 3). EMSA detected one specific DNA protein complex that was super-shifted by anti-c-Jun antibody but not by anti-c-Fos, suggesting that the AP1 DNA complex is composed mainly of a c-Jun homodimer. Formation of AP1 complex was not inhibited by a 100-fold molar amount of an oligonucleotide containing a Sp1 binding site but was completely inhibited by competition from a 100-fold molar amount of unlabeled AP1 probe (lane 2). The AP1-MU2 mutation totally lost the ability of the mutated oligonucleotide to compete for the binding, whereas the binding capacity of the oligonucleotide AP1-MU1 was decreased to 80% of the wild-type sequence, suggesting that the change of nucleotides on AP1-MU1 has a less damaging effect on AP1 binding than that of AP1-MU2. Taken together, these results demonstrate that AP1 motifs located on the sense strand and the antisense strand of intron 1 are both required for BCSG1 transcription.

We were interested in determining whether the intronic AP1 sites are involved not only in the basal transcription but also participate in the regulated transcription of BCSG1 by agents that induce AP1 transactivation. Because TPA is a well known AP1 activator (15, 16), we examined the effects of TPA on BCSG1 promoter activity. Treatment of cells with TPA for 24 h stimulated the BCSG1 promoter activity 3.1-fold in SKBR-3 cells, 2.3-fold in T47D, and 4.9-fold in HepG2 cells. Mutation of the AP1 site (AP1-MU1) completely abolished induction (Fig. 4A). EMSA showed that the elevated BCSG1 promoter activity was accompanied by increased AP1 DNA binding activity at the intronic AP1 sites in TPA-treated SKBR-3 and T47D cells (Fig. 4B). The stimulating effect of TPA on BCSG1 transcription was confirmed further by measuring the endogenous BCSG1 mRNA. RT-PCR analysis showed that TPA treatment increased BCSG1 mRNA 2.1-fold in SKBR-3 cells, whereas the mRNA level of GAPDH was not altered (Fig. 4C).

To demonstrate that the induction of BCSG1 transcription by TPA is the direct result of increased c-Jun binding to the intronic AP1 sites under in vivo conditions in intact cells, we performed the ChIP assays, which directly examined the binding of c-Jun to the chromatin region encompassing the BCSG1 intronic AP1 sites. Control and TPA-stimulated SKBR-3 cells were treated briefly with formaldehyde to cross-link DNA-binding proteins to chromatin. The isolated chromatin was subjected to sonication followed by immunoprecipitation with rabbit anti-c-Jun antibody or rabbit normal IgG as a negative control for nonspecific binding. DNA from the immunoprecipitate was isolated. From this DNA, a 200-bp fragment of the
BCSG1 intron 1 region surrounding the AP1 sites was amplified. Fig. 5A shows that the level of c-Jun cross-linked to the BCSG-AP1 sequence was increased 5-fold in TPA-stimulated cells as compared with the control after normalization with the input DNA.

To further examine the effect of AP1 mutation on TPA-induced binding of c-Jun to this intron 1 region in vivo, SKBR-3 cells were transiently transfected with BCSG1 promoter luciferase construct BCSG1967AP1-MU2. The transfected cells were untreated or treated with TPA for 24 h. The ChIP assays were conducted using two sets of primers, which amplified the plasmid DNA and the endogenous DNA separately. To amplify the intronic AP1 sites from the plasmid DNA, the primers AP1CHIP-5' and AP1CHIP-3'E were used, which yielded a 240-bp PCR product containing the BCSG1 sequence (+549 to +707) plus 80-bp vector sequence. As shown in Fig. 5B, whereas the binding of c-Jun to the endogenous BCSG1 intronic AP1 region was increased 3.6-fold by TPA, the binding of c-Jun to the transfected DNA containing the mutated AP1 site was not significantly increased in TPA-treated cells. The results of ChIP assays clearly confirmed our in vitro studies, demonstrating that the interaction of c-Jun with the intronic AP1 sites is important for the basal as well as TPA-induced transcription of the BCSG1 gene in breast cancer cells.

To obtain direct functional evidence that AP1 controls BCSG1 transcription, we employed the approach of the dom-
in TAM67 cells with that in mock-transfected cells. Fig. 7

expression in TAM67 cells were decreased to levels below de-
tectable. Western blot using specific anti-BCSG1 antibody shows that the amounts of BCSG1 protein
transfected cells in soft agar. Fig. 8 shows that after 7 days of

TAM67, demonstrating that BCSG1 transcription in TAM67
cells was strongly inhibited. Western blot analysis using an anti-c-Jun antibody shows that the
29-kDa c-Jun mutant was expressed only in TAM67 clones
and selection of resistance to neomycin G418, several stable
clones were established along with the control clones that
were transfected with the empty vector pcDNA3.1. Western
blot analysis using an anti-c-Jun antibody shows that the
100-fold of molar amounts of unlabeled competitor DNA (lanes 2–5) or
in the presence of antibodies to c-Jun (lane 6) or c-Fos (lane 7). The
reaction mixtures were loaded onto a 6% polyacrylamide gel and run in
TGE buffer at 180 V for 2.5 h at 4°C. An EMSA using nuclear extract
of T47D or other breast cancer cell lines showed similar results.
instant-negative mutant to inhibit the AP1 transcriptional
activity. TAM67 is a deletion mutant of c-Jun that lacks the
transactivation domain. TAM67 has been demonstrated to act
as a potent inhibitor of AP1 transactivating activity by
forming homodimers with full-length c-Jun and heterodimers
with c-Fos and to quench the activity of AP1 complexes (17).
After transfection of pcDNA-TAM67 vector into T47D cells
and selection of resistance to neomycin G418, several stable
clones were established along with the control clones that
were transfected with the empty vector pcDNA3.1. Western
blot analysis using an AP1 luciferase reporter, pAP1-Luc, which contains
seven AP1 sites upstream of a basal promoter (Stratagene).
Fig. 6B shows that compared with T47D and mock-trans-
fected cells, the AP1 promoter activities in all three TAM67
clones were reduced to nearly the base-line level. These data
clearly demonstrate that the AP1 transcriptional activity in
T47D cells was greatly suppressed by expression of TAM67.
Thus, RT-PCR assays were conducted to detect BCSG1
mRNA in TAM67 cells and in the control cells. Fig. 7A shows that a single band of 337 bp corresponding to the coding
region of BCSG1 was readily detected in T47D cells as well as
in the cells transfected with the empty vector. In contrast,
this band was nearly undetectable in all clones that express
TAM67, demonstrating that BCSG1 transcription in TAM67
cells was strongly inhibited. Western blot using specific anti-
BCSG1 antibody shows that the amounts of BCSG1 protein
expressed in TAM67 cells were decreased to levels below de-
tection (Fig. 7B). Moreover, we compared BCSG1 promoter activity
in TAM67 cells with that in mock-transfected cells. Fig. 7C shows that the promoter activity of BCSG1967 was markedly decreased
in all three TAM67 clones as compared with the clones of empty
vector, whereas the promoter activity of BCSG1964, which does
not contain the intronic AP1 sites, was not significantly affected
by expression of TAM67. Taken together, these results provide
strong evidence that AP1 is a critical transcription factor for
BCSG1 transcription through interaction with its recognition
sequences residing in the first intron.

Previously we had shown that ectopic expression of BCSG1
in MCF-7 cells stimulated cell proliferation (6). To determine
the effect of inhibition of BCSG1 expression by blocking AP1
transactivation on cell growth, the growth rates of TAM67
cells were compared with untransfected T47D and the mock-
transfected cells in soft agar. Fig. 8 shows that after 7 days of
culture in soft agar, the number and size of the colonies in
cells expressing TAM67 were reduced to 10–20% of T47D
cells.

The loss of BCSG1 expression in T47D-TAM67 cells may not
solely account for the decreased cell growth in soft agar, as
expression of TAM67 could inhibit other AP1-regulated genes
that play roles in cell proliferation. To directly examine the effect
of BCSG1 expression on the anchorage-independent growth of
breast cancer cells, we constructed the vector pcDNA-BCSG1-As,
which expresses the 5’ portion of the BCSG1 mRNA in an anti-
sense orientation. As a negative control, the vector

FIG. 3. EMSA of nuclear proteins interacting with the intronic
AP1 motifs. A 30-bp double-stranded oligonucleotide, designated
BCSG-Ap1, was radiolabeled and incubated with 4 μg of nuclear extract
prepared from SKBR-3 cells in the absence (lane 1) or presence of
100-fold of molar amounts of unlabeled competitor DNA (lanes 2–5) or
in the presence of antibodies to c-Jun (lane 6) or c-Fos (lane 7). The
reaction mixtures were loaded onto a 6% polyacrylamide gel and run in
TGE buffer at 180 V for 2.5 h at 4°C. An EMSA using nuclear extract
of T47D or other breast cancer cell lines showed similar results.
pcDNA-BCSG1-S was also made, which expresses the same mRNA sequence in a sense orientation. The plasmids pcDNABCSG-A and pcDNABCSG-S were transfected separately into T47D cells, and stable clones were selected. Western blot analysis showed that the BCSG1 protein expression in the clones expressing BCSG1 antisense mRNA was significantly reduced to the levels of 25–40% of T47D (Fig. 9A), indicating that BCSG1 translation was subverted by the antisense mRNA. Soft agar colony assays demonstrated that the anchorage-independent growth of T47D cells expressing BCSG1 antisense mRNA was significantly suppressed to a similar extent as in TAM67 cells (Fig. 9B), whereas the growth rate of the clone expressing
BCSG1 sense mRNA was not statistically different from that in untransfected T47D cells.

**DISCUSSION**

Activator protein AP1 is mainly composed of c-Jun homodimers or c-Jun/c-Fos heterodimers. The AP1 complex mediates the transcriptional activation of a variety of genes through its specific binding to the DNA sequence TGACTCA, otherwise known as an AP1 site. It has been demonstrated that AP1 transcriptional activity is increased with the induction of the transformed phenotype and with neoplastic progression (8–10, 18, 19). In this study, we have demonstrated that AP1 has an overriding role in the control of BCSG1 transcription in breast cancer cells through specific interaction with two closely located AP1 sites residing in the first intron.

Although transcription of most of the mammalian genes is controlled by regulatory elements and enhancer sequences located in the 5′-flanking region, there are a number of genes that contain regulatory sequences in the first intron. For example, an active AP1 site present in the first intron of the p67 (phox) gene is critically important for its myeloid-specific expression (20). The lineage-specific expression of MRP14 gene is controlled by a potent enhancer element located in the first intron as well (21). The 5′ promoter region of BCSG1 contains two AP1-like sequences at regions −1194 to −1188 and −488 to −482 relative to the translation start site. However, deletion

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**Fig. 7. Expression of TAM67 inhibits BCSG1 expression.**

A, RT-PCR analysis to detect BCSG1 mRNA and GAPDH mRNA expression. B, Western blot analysis to detect BCSG1 protein expression. 50 μg of protein from each sample was separated by SDS-polyacrylamide gel electrophoresis, and the BCSG1 protein expression was examined by Western blot analysis using a rabbit polyclonal antibody specific to BCSG1. C, BCSG1 promoter analysis. BCSG1 promoter luciferase reporter BCSG1967 and BCSG1864 were transiently transfected into mock clones or TAM67 clones along with pRL-SV40 vector. 40 h after transfection, cells were harvested and dual luciferase activities were determined.
of these sequences did not affect the basal promoter activity of BCSG1, suggesting that they are not functionally involved in BCSG1 transcription (7). In contrast, the two intronic AP1 motifs appear to be critically important in BCSG1 transcription, as deletion or mutation to eliminate AP1 binding to these sites markedly reduced the basal- and TPA-induced promoter activities.

The direct effect of AP1 transactivation on BCSG1 transcription is demonstrated in T47D cells that express a dominant negative mutant of c-Jun, TAM67. Our supershifted EMSAs as well as ChIP assays show that c-Jun is the main protein component in the AP1 complex that binds to the intronic AP1 sites. Therefore, blocking AP1 transcriptional activity by expression of TAM67 should produce an inhibitory effect on BCSG1 transcription.

We compared the levels of BCSG1 mRNA and protein in three stable clones of TAM67 with untransfected T47D and three mock-transfected clones. We show that expressions of BCSG1 in three mock-transfected clones are similar to the parental T47D cells, whereas BCSG1 mRNA and protein expressions in TAM67 clones were almost undetectable, demonstrating that blockade of AP1 transcriptional activity has a profound negative impact on BCSG1 transcription.

Previous studies in other cell types with TAM67 expression have shown that blocking AP1 transactivation inhibits the anchorage-independent growth of tumor cells and also neoplastic transformation (8, 18). In this study, by conducting soft agar colony assays we demonstrated that the growth rates of TAM67 cells are much slower than mock-transfected and untransfected parental T47D cells. TAM67-mediated inhibition of the anchorage-independent growth of T47D cells occurred, at least in part, because of the decreased expression of BCSG1, and we showed that inhibition of BCSG1 expression by producing BCSG1 antisense mRNA led to a strong growth inhibition of T47D cells in soft agar as well. These results provide additional evidence to support the hypothesis that BCSG1 might be a proto-oncogene, the expression of which stimulates the growth and transformation of breast cancer cells (2, 3, 5, 6).

Many extracellular stimulus affect cellular functions...
through activation of AP1 transcriptional activity. AP1 motifs have been shown to be a converging site for several signal transduction pathways including ERK/MAP kinase, protein kinase C, and c-Jun N-terminal kinase pathways (22–24). In this study, we used TPA as a tool to explore the regulation of BCSG1 expression in breast cancer cells and we demonstrate that BCSG1 transcription was activated by TPA. Using both in vitro DNA binding assays and in vivo ChIP assays, we demonstrate that c-Jun, the major protein component of the AP1 DNA binding activity, interacts with the intrinsic AP1 sites, and this interaction is further stimulated by TPA. Previously, we have shown that BCSG1 transcription in breast cancer cell line H3922 was strongly inhibited by the cytokine oncostatin M (OM). It is tentatively to speculate that OM may affect BCSG1 transcription through the intrinsic AP1 sites.

The abnormal transcription of BCSG1 in breast cancer cells is likely controlled by multiple mechanisms. The results presented herein clearly demonstrate that AP1 is a key positive regulator for BCSG1 transcription in cells such as T47D or SKBR-3 in which the BCSG1 gene is unmethylated. We have found that in MCF-7 or HepG2 cells, where BCSG1 gene is methylated, activation of AP1 transactivation only increased the BCSG1 promoter reporter activity without a positive effect on the endogenous gene (data not shown). The transcription initiation sites of BCSG1 gene are embedded in the CpG island; methylation in this region inhibits the interaction of basal transcriptional machinery with the promoter DNA and prevents the initiation of transcription. Thus, it is very likely that demethylation of the CpG island occurs in breast cancer cells as the first event, which allows BCSG1 to be transcribed. The abundant level of AP1 activity in tumor cells pushes the transcription of BCSG1 to a higher level.

BCSG1 expression is strongly associated with breast cancer disease progression and metastasis. When over-expressed, BCSG1 stimulates the proliferation and invasion of breast cancer cells. Our finding that AP1 is a key activator for BCSG1 transcription provides insightful information for further investigations to identify the different signaling pathways that lead to the activation of the BCSG1 gene. Elucidation of the molecular mechanisms underlying BCSG1 transcription is important for the development of therapeutic approaches to block BCSG1 expression and to reverse the malignant phenotypes.

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