Insulin-like growth factor I differentially regulates the expression of HIRF1/hCAF1 and BTG1 genes in human MCF-7 breast cancer cells

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Abstract. Differential display PCR analysis (DD-PCR) was used to identify novel genes that respond to IGF-I treatment in human MCF-7 breast cancer cells. Fifty-three cDNAs showed alterations in their mRNA levels in IGF-I treated cells. One of these genes showed a significant increase in the mRNA level in IGF-I treated cells in comparison to non-treated cells. We named this gene HIRF1 (human IGF-I regulated factor 1). Nucleotide blast analysis revealed that this gene has a 100% sequence identity with the sequence for BTG1 (B-cell translocation gene) binding factor 1 (human CCR4-associated factor 1 gene, hCAF1). By alignment of cloned HIRF1 cDNA and genomic DNA 8p21.3-p22 sequence, we were able to determine the exon-intron structure of the cloned HIRF1 gene on chromosome 8. Northern blot and real-time PCR analysis showed that BTG1 and c-fos reached their maximal expression fairly early within 10 min to 1 h, and decreased to basal levels after 3 h of IGF-I treatment. HIRF1/hCAF1 expression reached maximal stimulation after 3 h of IGF-I treatment and then gradually decreased to basal level. HIRF1 and BTG1 mRNA was inhibited by inhibitors of the cell signaling pathways, PI3/Akt kinase and MAPK kinases (ERK1/2 and p38). In summary, cloned HIRF1/hCAF1 is co-regulated with BTG1 in response to IGF-I. The regulation of these genes as early response genes may have an important role in differentiation, growth and proliferation of breast cancer cells.

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Introduction

The multi-step process of breast carcinogenesis involves various genetic and phenotypic alterations. Cellular processes such as cell growth, cell cycle regulation, cell metabolism, signal transduction, and apoptosis are deregulated. Addition of a mitogen such as insulin-like growth factor I (IGF-I) to the breast tumor-derived MCF-7 cell causes stimulation of cell cycle progression. Consequently, the genes critical for G1/S, or/and G2/M transitions must be differentially expressed upon IGF-I treatment. However, in breast cancer cells, the identity and the molecular mechanisms of these IGF-I responsive genes during cell cycle progression have yet to be fully characterized.

The regulation of processes such as cell growth, development, differentiation, apoptosis, cell cycle progression and carcinogenesis involves a balance between proto-oncogenes and tumor suppressor genes. The expression of these genes is affected by growth factors that bind to cell-surface receptors and activate or deactivate specific target genes through secondary messenger systems. Changing the normal expression pattern of these genes can lead to uncontrolled cell proliferation, cell cycle arrest or apoptosis (1).

The insulin-like growth factors (IGF-I and IGF-2) are mitogens for breast cancer cells (2). A number of studies have shown that the proliferative effect is mediated predominantly by insulin-like growth factor 1 receptor (IGFR-1). IGF-I initiates its growth-promoting effects through its transmembrane tyrosine kinase receptor (3). Upon activation by ligand binding, the IGF-1R tyrosine kinase phosphorylates several intracellular substrates, such as the insulin receptor substrate (IRS) proteins (IRS-1 through -4) and Shc. In breast cancer cell lines that express estrogen receptors (MCF-7, T47-D), the mitogenic effects of IGF-I are primarily mediated by IRS-1. Activated IRS-1 subsequently regulates proteins with SH2 homology, including the p85 regulatory subunit of phosphatidylinositol 3'-kinase (PI3K) and the adapter protein, Grb2 (4-7). Some of the downstream effectors of PI3K are the serine/threonine protein kinase, Akt/PKB, and the p70/S6 kinase (8,9). The binding of IRS and Shc proteins to Grb2 and the associated guanine nucleotide exchange protein, mSos,

result in activation of the Ras-Raf-MAP kinase pathway. The specific pathway involved in cell proliferation (i.e. PI3K versus MAP kinase) depends on the particular cell type. In MCF-7 cells, the proliferative response to IGF-I is mediated by PI3K. More specifically, it has been shown that, in MCF-7 cells, the PI3K pathway is involved in cyclin D1 synthesis and the hyperphosphorylation of the retinoblastoma protein (Rb) (10). Further studies have shown that Akt affects cyclin D1 translation in response to serum in MCF-7 cells. The multitude of genes activated by IGF-I in breast cancer cells has not been completely understood.

CAF1 (carbon catabolite repressor protein 4 - associative factor 1) has been implicated to play an important role in control of transcription and mRNA decay. The CAF1/POP2 protein has been best characterized in *S. cerevisiae*. The yeast, CAF1/yPOP2, and its partners, CCR4 and NOT proteins, form a gigantic complex. The CCR4-CAF1-NOT complex regulates a number of genes, both positively and negatively, via different pathways, including control of transcription and mRNA decay (11-14). The CCR4-CAF1-NOT complex regulates the transcription of diverse genes through contacts made with TFIID, the SAGA complex and SRB9-11 protein (15-17). The role of CAF1 in the complex is not defined clearly; it may act as a bridge between CCR4 and NOT proteins (18). CAF1 and CCR4 also function as cytoplasmic deadenylases in yeast and *Drosophila* (19,20). In yeast, additional factors, such as CAF16, and CAF4 are found to be present in the larger 1.9 MD CCR4-CAF1 complex and affect transcription both positively and negatively (21).

The family of CAF1 protein complexes is involved in the control of transcription events. Furthermore, database analysis also showed that nucleotide HIRF1 sequence has 100% sequence identity with several regions of *Homo sapiens* chromosome 8 short arm DNA segment. The chromosome 8p21.3-p22 region was previously identified as an anti-oncogene of hepatocellular, colorectal and non-small cell lung cancer. Previously, hCAF1 was isolated by the yeast 'two-hybrid' system as an interacting protein partner of the BTG1, potential tumor suppressor gene. Others postulated that formation of the hCAF1/BTG1 complex possibly interferes with intracellular signal pathways of cell division events.

The CAF1-CCR4-NOT complex is structurally conserved among all higher eukaryotes. Two homologs of yCAF1/yPOP2, hCAF1 and CALIF, have been identified in humans (22,23). Additionally, CAF1 from human, mouse and *Drosophila* has been shown to associate with members of the BTG/TOB protein family (24-26). The BTG family of proteins are negative regulators of the cell cycle, their molecular role being not fully understood (27,28). The human BTG1 protein is thought to be a potential tumor suppressor because its overexpression inhibits NIH 3T3 cell proliferation. The BTG1 gene contains a long 3' AU - rich untranslated region, characteristic of unstable mRNA species, such as those found in cell cycle control genes (29). Furthermore, the mature BTG1 protein contains PEST residues (Pro, Glu, Ser and Thr) characteristic of highly unstable proteins including cyclins and transcription factors (20,31,32). The mCAF1 protein, along with BTG1 and BTG2, has been shown to control estrogen receptor α-dependent transcription, both positively and negatively. The CCR4-NOT1-CAF1 complex is likely to have a critical role in cellular homeostasis.

In this study, DD-PCR analysis identified human IGF-I regulated factor 1 (HIRF1)/hCAF1 up-regulated in human MCF-7 breast cancer cells. To understand the role of IGF-I induced expression of the HIRF1 gene in breast cancer cells, we examined the HIRF1, BTG1, and c-fos mRNA expression levels in synchronized MCF-7 cells after IGF-I induction. Northern blot data showed that HIRF1 expression reached the maximum 3 h after IGF-I induction and then it gradually decreased to basal levels. Interestingly, BTG1 reached its maximal expression within 5 min of IGF-I treatment and soon after returned to basal levels.

We proposed that the cloned HIRF1/hCAF1 gene from breast cancer cells is identical to the previously cloned BTG1 binding factor 1 or hCAF1 gene. HIRF1/hCAF1 is located at the short arm of human chromosome 8p21.3-p22 anti-oncogene segment. Northern blot analysis suggested that, in IGF-I induced human breast cancer MCF-7 cells, differential expression of closely related HIRF1 and BTG1 genes as well as hCAF1/BTG1 complex formation could be highly controlled by IGF-I. Real-time PCR analysis demonstrated that both HIRF1 and BTG1 are regulated by IGF-I in a time-dependent manner. The regulation of both genes is dependent on the activation of MAPK. Inhibitors of MAPK (ERK1/ERK2) and p38 MAPK abrogated the IGF-I induced activation of HIRF1 and BTG1.

Materials and methods

Cell culture. One hundred mm tissue culture dishes of human breast cancer MCF-7 cells were grown to 90% confluence in DMEM-F12 containing 10% fetal bovine serum (FBS) media. Cells were incubated overnight in DMEM-F12/0.5% FBS-12 for synchronization. Next day, from one set of dishes, RNA was isolated and purified immediately after synchronization. This set was designated as control. The second set of dishes were incubated for 18 h in media with dialyzed serum DMEM/5% DFBS-12 (-AA), and the third set of dishes were incubated for 18 h in media with dialyzed serum supplemented with 100 ng/ml of human IGF-I (R&D) (-AA, +IGF-I). RNA was purified using RNAzol B reagent (TelTest Inc.)

Differential display PCR (DD-PCR). Modified DD-PCR was performed using the Delta RNA Fingerprinting kit (Clontech, Palo Alto, CA), based on an improved method described by McClelland *et al* (33). Two μg of total RNA from three conditions [MCF-7 control, MCF-7 treated for 18 h in dialyzed serum (-AA) media and MCF-7 treated for 18 h in -AA, +IGF-I 100 ng/ml] were reverse-transcribed with 200 units of SuperScript II RT enzyme (Gibco-BRL Life Technology Inc., Bethesda, MD) in the presence of 1 μM oligo(dT) primer for 1 h at 42˚C in a total volume of 10 μl. The reaction was terminated by incubation at 75˚C for 10 min. After first-strand cDNA synthesis, 2 μl and 8 μl of the reaction product was diluted to a 100 μl total volume and then 1 μl of each dilution was used in a separate PCR reaction. Each 20 μl PCR reaction contained 1 μl of cDNA dilution, 1 μM arbitrary P primer, 1 μM anchored T primer, 1X Advantage KlenTaq Polymerase Mix (1% glycerol, 0.8 mM Tris-HCl pH 7.5, 1 mM

KCl, 0.5 mM $(NH_4)_2SO_4$, 2 mM EDTA, 0.1 mM ß-mercapto-ethanol, 0.005% Thesit, KlenTaq-1 DNA polymerase, $Vent_R$ polymerase, and TaqStart antibody (1.1 μg/μl), 1X KlenTaq PCR reaction buffer (40 mM Tricine-KOH pH 9.2 at 25˚C, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, and 75 μg/ml BSA), 50 mM dNTP, and 50 nM of [α-^{33}P]dCTP (1,000-3,000 Ci/mmol) (Amersham Life Sciences, Arlington Heights, IL). After amplification at 94˚C for 5 min; 40˚C for 5 min and 68˚C for 5 min for 1 cycle; 94˚C for 30 sec, 40˚C for 30 sec and 68˚C for 5 min for 2 cycles; and 94˚C for 20 sec, 60˚C for 30 sec and 68˚C for 2 min for 30 cycles; we performed an extension at 68˚C for 7 min on a GeneAmp PCR system 9600 (Perkin-Elmer Corp., Norwalk, CT). Each PCR reaction was stopped by the addition of Stop Solution (Amersham). Samples were heated at 94˚C for 2 min and analyzed on a denaturing 4.5% polyacrylamide gel using a GenomyxLR™ DNA Sequencer (Genomyx, Foster City, CA).

Sequencing of cloned cDNAs and data analysis. The cDNA fragments showing significant fold induction after IGF-I treatment were cloned into the pCRII vector using the TA vector cloning system (Invitrogen, Carlsbad, CA). Sequence analysis was performed using M13 forward and reverse primers with a fluorescent automated 373 DNA sequencer (Applied Biosystems, Foster City, CA). The sequenced cDNAs were analyzed via the BLAST program for matches in the GenBank database and compared with each other via FASTA analysis.

Northern blot analysis. Samples containing 20 μg of total RNA were fractionated on a 0.8% agarose gel containing 2.2% formaldehyde and 1X MOPS (50 mM MOPS, 1 mM EDTA) (Oncor, Gaithersburg, MD) and transferred to a nylon membrane (Stratagene, La Jolla, CA). The membranes were cross-linked using a UV Crosslinker (Fisher Scientific) and hybridized with labeled probe. The probe used in Northern blot analysis was prepared by *Eco*RI/*Ssp*I digestion for the 'cloned' cDNA probe [605 base pair fragment that begins at nucleotide 132 and ends at nucleotide 737 using [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham) and a High Prime DNA Labeling kit (Boehringer Mannheim)]. Hybridization was performed for 2 h at 68˚C using QuikHyb hybridization solution (Stratagene). Hybridization signals were quantitated by a Densitometer 300A (Bio-Rad, Hercules, CA) and normalized by corresponding GAPDH signals.

Gene cloning and characterization. TA cloning (Invitrogen, Carlsbad, CA) ligated the DD-PCR product into pCR2.1 vector and cloned cDNA was sequenced from both directions. The ORF finder in the Web pages of the National Center for Biotechnology Information (NCBI) deduced the amino acid sequence of HIRF1 (www.ncbi.nlm.nih.gov/gorf/gorf.html). The functional domains of HIRF1 were predicted using the Predict Protein server in the EMBL Web pages (www.embl-heidelberg.de/predictprotein/predictprotein.html). The alignment of the HIRF1 protein sequence with 5 other CAF family members was performed using the Clustal W 1.7 multiple sequences alignment program at the Baylor College of Medicine website BCM Search Launcher. BLAST search of the HIRF1 gene indicated 100% homology to hCAF1, located on 8p21.3-p22 (accession number: AB020860). By alignment of two sequences, we were able to determine the exon-intron structure of the HIRF1 gene.

In vitro transcription and translation. *In vitro* transcription and translation analysis of HIRF1 gene expression was performed using the TNT® T7 quick coupled transcription/translation system (Promega, Madison, WI) according to the manufacturer's instruction. Briefly, 1 μg of pCR2.1-HIRF1 and pCR2.1-p21/WAF1 (as control) was used in the standard 50-μl reaction in the presence of ^{35}S-methionine and was incubated at 30˚C for 90 min. The reaction was resolved in 12.5% of SDS-PAGE gel and the gel was vacuum dried and exposed to the Fuji film overnight.

Total RNA isolation and real-time quantitative PCR. Total cellular RNA from MCF-7 cells, the synchronized control and cells treated with IGF-I were isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen, San Diego, CA). Approximately 3 μg of each RNA sample was used in a first-strand cDNA synthesis reaction (containing RNase inhibitor) using a standard poly-dT adapter primer, random-hexamer primer and thermoscript reverse transcriptase (Thermo-Script RT-PCR system, Invitrogen, San Diego, CA). Samples were incubated for 60 min at 50˚C and heated at 85˚C for 5 min to inactivate the reverse transcriptase. Aliquots (2 μl) of the RT reaction mixture were then amplified using a Quanti Tech SYBR-Green PCR kit (Qiagen, Valencia, CA) containing specific primer sets for BTG1, HIRF1 and a housekeeping gene, 18S RNA. The primers used were BTG1 (150 bp), 5'-TGT TCA GGC TTC TCCCAAGT-3' (sense) and 5'-CTA CCA TTT GCA CGT TGG TG-3' (antisense); HIRF1/hCAF1 (141 bp), 5'-GGA ACG GAT AGG ACC ACA AC-3' (sense) and 5'-AGG ATG AAC CAG AAC CAA GG-3' (antisense).

PCR and the monitoring of the PCR products were performed using an ICyclerIQ (Bio-Rad). Quantitative analysis of gene expression was performed using comparative C_T (ΔC_T) methods, in which C_T is the threshold cycle number (the minimum number of cycles needed before the product can be detected). The arithmetic formula for the ΔC_T method is the difference in threshold cycles for a target (i.e., HIRF1/hCAF1) and an endogenous reference (i.e., housekeeping gene 18S). The amount of target normalized to an endogenous reference (i.e., HIRF1/hCAF1 in IGF-I induced cells) and relative to a calibration normalized to an endogenous reference (i.e., HIRF1/ hCAF1 in synchronized cell controls) is given by $2^{-\Delta\Delta CT}$.

Effects of Ly294002 or PD98050, SB202190 on HIRF1 and BTG1 mRNA expression in MCF-7 breast cancer cells. MCF-7 cells at 75% were synchronized overnight with DMEM/F12 media containing 0.5% dialyzed FBS (10,000 kDa cut-off). Cells were pretreated with 50 μM Ly29402, 30 μM PD98050 or 10 μM and 30 μM SB202190 for 60 min and then stimulated with 50 ng/ml IGF-I (+) or without IGF-I (-) for 3 h. Inhibitors were present throughout the incubation. Total RNA was extracted and quantitative RT-PCR was performed as outlined above. Each value represents the mean of duplicate determinations.

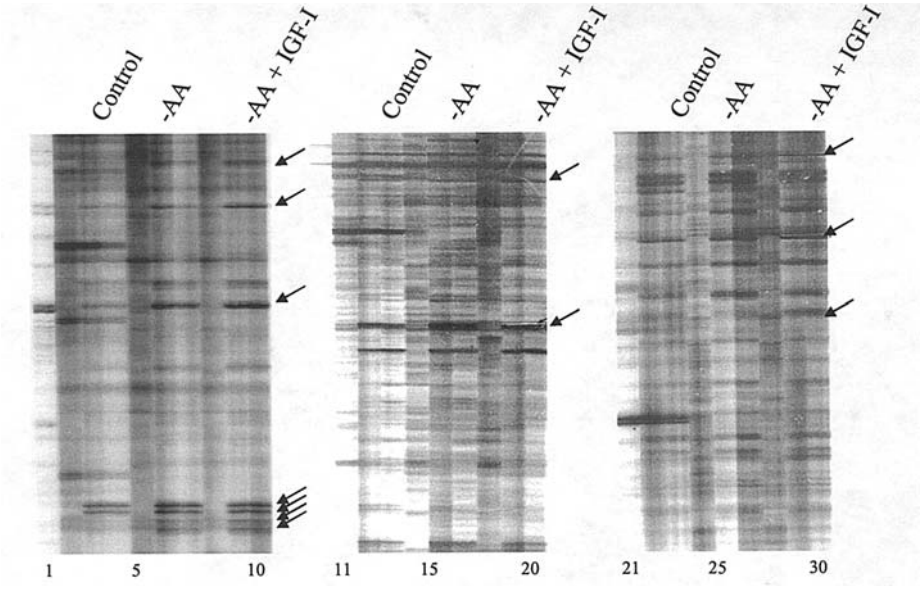

Figure 1. DD-PCR gels showing differentially expressed bands/genes. Representative patterns of differentially expressed genes using three different primer combinations are shown (lanes 1-10, 11-20, 21-30). Control, minus amino acids (-AA) and minus amino acid plus IGF-I (-AA + IGF-I) treatment are the three different conditions in MCF-7 cells used in DD-PCR analysis. The arrows indicate the bands that are up-regulated after IGF-I treatment from DD-PCR gels using two different cDNA concentrations (compare lanes 9/10, 19/20, and 29/30 to lanes 6/7, 16/17, and 26/27). Control DD-PCR without the reverse-transcriptase reaction is shown in lanes 2, 12, and 22 using control MCF-7 RNA, lanes 6, 16, and 26 using -AA MCF-7 RNA, and lanes 7, 17, and 27 using -AA + IGF-I MCF-7 RNA. Lanes 1, 11 and 21 represent water controls for every primer combination.

Results

Isolation of differentially expressed cDNAs by DD-PCR in IGF-I-treated MCF-7 cells. A modified DD-PCR method was used to identify genes uniquely and/or highly expressed in human breast cancer MCF-7 cells in response to IGF-I treatment. For example, a DD-PCR gel using three different primer combinations is shown in Fig. 1. Approximately 80 bands were generated (sizes ranging between 300 bp and 2.0 kb) for each DD-PCR reaction. The majority of the bands were the same intensity but only the cDNA fragments, which were up-regulated upon IGF-I induction, had a higher intensity and they are indicated with arrows (Fig. 1, marked arrows in lanes 9/10, 19/20 and 29/30). Overall, among approximately 6,000 generated DD-PCR bands, 53 cDNA patterns were reproducibly obtained as more abundant in IGF-I-induced MCF-7 cells. All 53 up-regulated cDNA bands were isolated from DD-PCR gels and re-amplified by PCR in the presence of original primers. For each fragment, 10 μl aliquots were run on an agarose gel and confirmed that the molecular size of the re-amplified fragments corresponds to the size obtained by DD-PCR analysis (data not shown).

Fragment #4 is overexpressed upon IGF-I induction. To confirm the changes in gene expression and to eliminate false-positive clones, Northern blot analysis was performed to measure the respective mRNA levels between the control and IGF-I-treated cells. One of the probes, fragment #4, approximately 1 kb long, showed increased mRNA expression in IGF-I-induced cells (Fig. 2). We named this gene human IGF-I regulated factor 1 (HIRF1).

The IGF-I-induced HIRF1 gene has high sequence identity with human CAF1. To obtain the full-length sequence for fragment #4, two independent colonies containing HIRF1

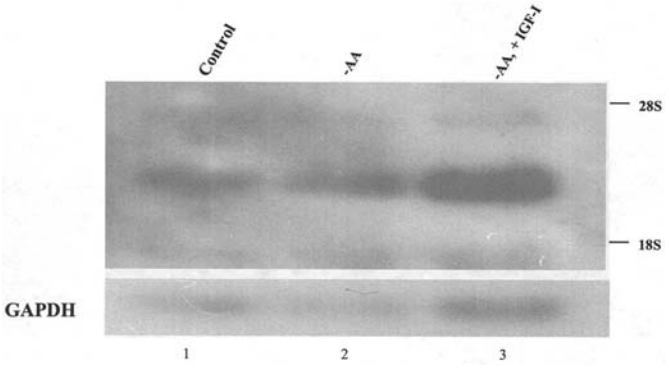

Figure 2. HIRF1/hCAF1 mRNA levels in three MCF-7 cell conditions. Total RNA (20 μg) from control (1), -AA (2) and -AA, + IGF-I (3) MCF-7 cells were analyzed by Northern analysis using a labeled HIRF1 probe. The size of 28S and 18S rRNA are indicated on the right. The membrane was re-hybridized with labeled GAPDH probe and is shown on the bottom panel.

cDNA fragments were sequenced with M13 forward and reverse primers. From both clones, the identical sequence of 976 bp nucleotides was obtained. This sequence was then submitted to the BLAST nucleotide databases and compared with previously published sequences. There was 99% sequence identity with BTG1 binding factor 1 (hCAF1) mRNA (gbL46722.1), 93% sequence identity with Mus musculus mCAF1 mRNA (gbU21855); 100% sequence identity with *Homo sapiens* genomic DNA of 8p21.3-p22 anti-oncogene of hepatocellular, colorectal and non-small cell lung cancer segment 3/11 (dbjAB020860), and 78% homology with human POP2 mRNA (gbAF053318). Furthermore, sequence analysis showed that the 976-bp fragment contained several open reading frames (ORF). The longest ORF begins at the start codon at position 44 (Fig. 3,

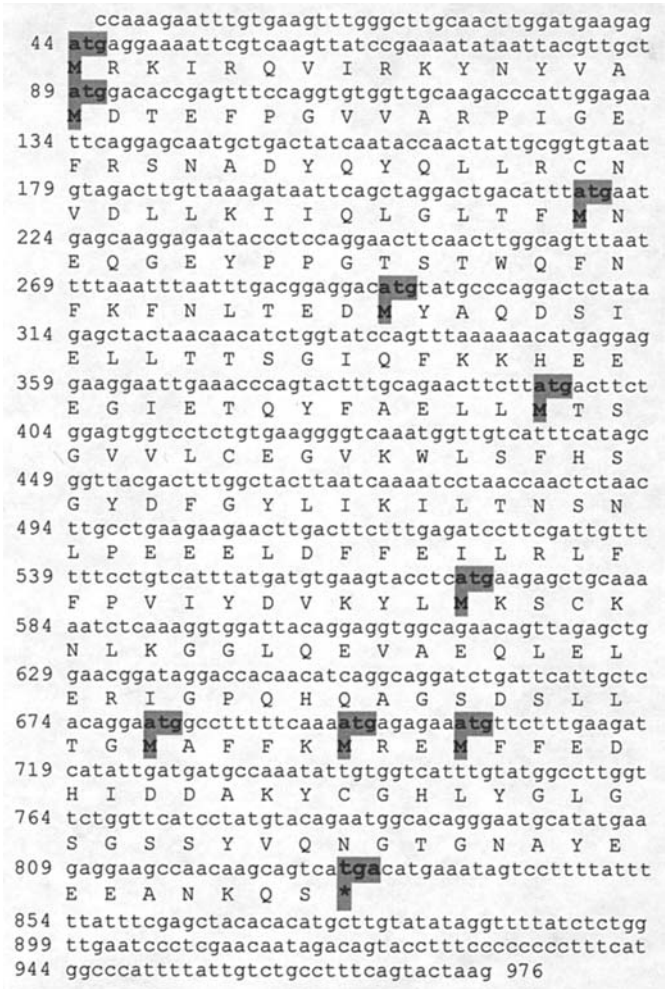

Figure 3. Nucleotide and predicted amino acid sequence of the open reading frame of the HIRF1/hCAF1 gene. The HIRF1 open reading frame begins at the start codon at position 44 and ends at the stop codon at position 830. The predicted length of the HIRF1 protein is 262 amino acids. One-letter code is used for the amino acid sequence. The probe used in Northern blot analysis was prepared by *Eco*RI and *Ssp*I digestion of the HIRF1 cDNA clone which corresponds to a 605-bp fragment that begins at nucleotide 132 and ends at nucleotide 737.

highlighted) and ends at the stop codon at position 830 (Fig. 3, highlighted) which potentially could encode a 262 amino-acid protein. The predicated functional sites of the protein are as follows: NLTE is a potential N-glycosylation site; RSNADYQY is a potential tyrosine kinase phosphorylation site; SNAD is a potential casein kinase phosphorylation site; GVVLCE is a potential N-myristylation site, and SCK is a potential protein kinase C phosphorylation site (Fig. 3).

When the sequence of the HIRF1 predicted protein was submitted to the BLAST protein database, the sequence showed 100% amino acid sequence identity to human CCR4-associated factor 1 protein (hCAF1; gbAAF01500.1), 99% identity to mouse CCR4-associated factor 1 (mCAF1; spQ60809), 73% identity to human POP2 (gbAAD02685), 58% identity to putative transcription factor (CCR4-associated factor homologue) from *Scizosaccharomyces pombe* (embCAA21420), and 38% identity to POP2 protein from *Saccharomyces cerevisiae* (dbjBAA02247). Alignment of amino acid sequence homology between HIRF1 and CAF family proteins is shown in Fig. 4. Fig. 5 shows the predictive intron-exon structure of the cloned HIRF1/hCAF1.

In vitro expression of the HIRF1/hCAF1 protein. In order to confirm that the HIRF1/hCAF1 cDNA ORF produces the predicted 262 amino-acid HIRF1/hCAF1 protein, an *in vitro* transcription and translation assay was performed. SDS-PAGE analysis showed an HIRF1/hCAF1 protein product band with an apparent molecular mass of 30 kDa (Fig. 6, lane 1).

Expression of HIRF1/hCAF1, BTG1 and c-fos on IGF-I induction of MCF-7 cells. IGF-I differentially regulates the expression of BTG1 and HIRF1/hCAF1 genes. Northern blot analysis was performed to measure the mRNA levels for HIRF1/hCAF1, BTG1 and c-fos after different time points of IGF-I induction. In response to IGF-I, HIRF1 expression was monitored for 5 min, 30 min, 1, 3, 5, 8, and 18 h after stimulation with IGF-I. Northern blot data showed that HIRF1/hCAF1 expression was maximal 3-5 h after IGF-I treatment. In contrast, the HIRF1/hCAF1 closely associated BTG1 gene, as well as c-fos, was maximally stimulated as early as 30 min to 1 h after IGF-I treatment. Our data suggest that IGF-I regulates HIRF1, and BTG1 gene expression in breast cancer cells. The c-fos gene expression was tested as an example of an early response gene.

To verify the effect of IGF-I on HIRF1/hCAF1 and BTG1 transcript levels (relative to those of 18S), we assayed mRNA levels by quantitative RT-PCR. MCF-7 cells were stimulated with IGF-I for different periods of time. As shown previously (Fig. 7), Northern blots showed a temporal control level of both BTG1 and HIRF1/hCAF1 RNA. Stimulation of MCF-7 cells with IGF-I (50 ng/ml) resulted in significant increases in HIRF1/hCAF1 mRNA levels as early as 5 min after induction when compared to synchronized controls (Fig. 8A). Similarly, mRNA levels for BTG1 were also significantly elevated, with a sharp increase at 5 min noted in IGF-I-treated MCF-7 cells as compared to controls (Fig. 8B). HIRF1 and BTG1 mRNA were barely detectable in synchronized cells; however, both HIRF1 and BTG1 mRNA expression were markedly up-regulated by 5 min, with a significant decrease in expression at 30 min of IGF-I exposure. For HIRF1, maximal expression occurred at 3 h and declined by 18 h (Fig. 8A and B). Levels of BTG1 showed a very sharp increase at 5 min (25-fold) and later time points showed a smaller increase (varying 10- to 20-fold) in the induction of BTG1 following IGF-I stimulation. Thus, the quantitative RT-PCR data further strengthens our results with Northern blotting.

Effect of inhibitors of PI3 kinase, MAPK (ERK1/2) and p38 MAPK on IGF-I induced HIRF1 and BTG1 mRNA expression in MCF-7 cells. Next, we investigated whether the MAPK and PI3K/Akt pathways play a role in the activation of IGF-I-mediated induction of HIRF1/hCAF1 and BTG1 mRNA. Monolayer cultures were pretreated for 1 h with inhibitors of PI3/Akt (LY294002), MAPK ERK1/2 (PD98059), and p38 MAPK (SB202190). The data in Fig. 8C and D show that, compared to non-treated control cells, all three inhibitors significantly inhibited the IGF-I induced up-regulation of HIRF1/hCAF1 and BTG1 mRNA. However, the MAPK and p38 MAPK inhibitors were more potent. The PI3 kinase

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                1                                                   50
caf1_human MRKIRQVIRK YNYVAMDTEF PGVVARPIGE FRSNADYQYQ LLRCNVDLLK
caf1_mouse MKKIRQVIRK YNYVAMDTEF PGVVARPIGE FRSNADYQYQ LLRCNVDLLK
caf1_caeel FARIRGFVED YPYVAMDTEF PGVVATPLGT FRSKEDFNYQ QVFCNVNMLK
pop2_yeast FAVIRQLVSQ YNHVSISTEF VGTLARPIGT FRSKVDYHYQ TMRANVDFLN

               51                                                  100
caf1_human IIQLGLTFMN EQGEYPPGTS TWQFNFKFNL TEDMYAQDSI ELLTTSGIQF
caf1_mouse IIQLGLTFMN EQGEYPPGTS TWQFNFKFNL TEDMYAQDSI ELLTTSGIQF
caf1_caeel LIQVGFAMVN DKGELPPTGD VWQFNFNFSF AEDMFSHESV EMLRQAGIDF
pop2_yeast PIQLGLSLSD ANGNKPDNgs TWQFNFEFDP KKEIMSTESL ELLRKSGINF

               101                                                 150
caf1_human KKHEEEGIET QYFAELLMTS GVVLCEGVKW LSFHSGYDFG YLIKILTNSN
caf1_mouse KKHEEEGIET QYFAELLMTS GVVLCEGVKW LSFHSGYDFG YLIKILTNSN
caf1_caeel TLLQNNGIPT AVFGELLTTS GLITDPRITW LTFSSGYDFG YLLKSITLGD
pop2_yeast EKHENLGIDV FEFSQLLMDS GLMMDDSVTW ITYHAAYDLG FLINILMNDS

               151                                                 200
caf1_human LPEEELDFFE ILRLFFPVIY DVKYLMKSCK NLKGGLQEVA EQLELERIGP
caf1_mouse LPEEELDFFE ILRLFFPVIY DVKYLMKSCK NLKGGLQEVA EQLELERIGP
caf1_caeel LPKEESTFFM CHKTLFPTSF DIKILLRTPn kLKGGLQEVA DQLDVKRQGV
pop2_yeast MPNNKEDFEW WVHQYMPNFY DLNLVYKIIq qQQYSLTTLA DELGLPRFSI

               201                                                 250
caf1_human QHQAGSDSLL TGMAFFKMRE MFFEDHIDDA KYCGHLYGLG SGSSYVQNGT
caf1_mouse QHQAGSDSLL TGMAFFKMRE MFFEDHIDDA KYCGHLYGLG SGSSYVQNGT
caf1_caeel RHQAGSDALL TAATFFKIKK QFFGDNWNqp LICGHMFGLG SSLSLFHSSG
pop2_yeast FTTTGGQSLL MLLSFCQLSK lkFPNGTDFA KYQGVIYGID GDQ.......

               251       262
caf1_human GNAYEEEANK QS
caf1_mouse GNAYEEEASK QS
caf1_caeel STSRLGDETP QG
pop2_yeast .......... ..
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Figure 4. The amino acid sequence homology between the cloned HIRF1/hCAF1 gene and CAF family proteins. The predicted HIRF1/hCAF1 amino acid sequence was aligned with three other CAF-related protein sequences from different species reported in GenBank and SWISS-PROT by Lustal W multiple sequences alignment program. The three CAF-related protein sequences are as follows: 2, BTG1 binding factor 1 (hCAF1), accession number gb AAF01500.1; 3, mouse CCR4-associated factor 1 (mCAF1), accession number spQ60809; 4, human POP2 protein, accession number gbAAD02685. Amino acids conserved between the four sequences are shaded gray.

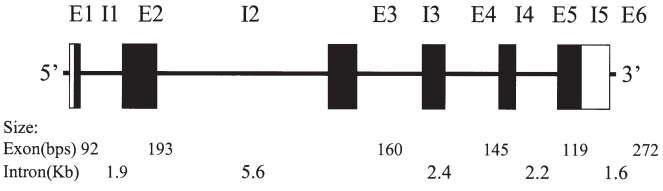

Figure 5. Exonic structure of the cloned HIRF1/hCAF1 gene. The exonic structure of the cloned human CAF gene was determined by alignment of cloned HIRF1 cDNA and its reported genomic sequence data in GenBank (accession number: AB020860). Exons (E) are shown as rectangles with introns (I) and flanking regions shown as lines. Open rectangles denote untranslated exons and shaded rectangles denote translated exons. The sizes of exons (base pairs, bps) and introns (kilobase pairs, kb) are indicated.

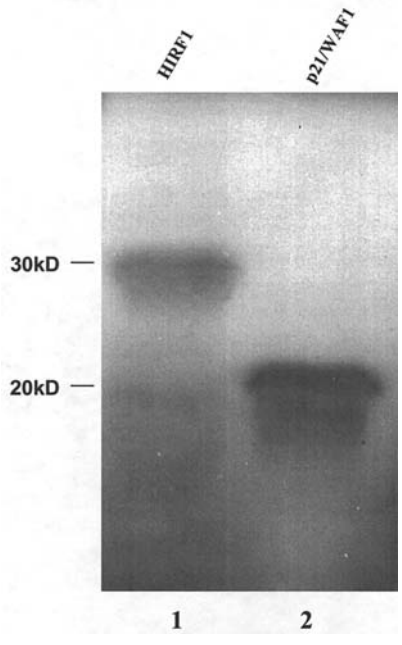

Figure 6. *In vitro* transcription and translation of the cloned HIRF1/hCAF1 gene. One μg of plasmid DNA, pCR2.1-HIRF1 and pCR2.1-p21/WAF1, was used for *in vitro* transcription and translation by TNT T7 quick coupled transcription/translation system. Lane 1, HIRF1; lane 2, p21/WAF1 as positive control.

inhibitor, LY294002, caused slightly less inhibition. IGF-I is known to induce MCF7 cells through both PI3 kinase and MAPK pathways (10,34).

We also investigated whether JNK or p38 pathways were involved in the IGF-I-mediated increase in HIRF1/hCAF1 and BTG1 genes. To determine whether inhibition of JNK and/or p38 abrogated the induction of HIRF1/hCAF1, inhibition experiments were performed with different concentrations of SB202190. MAPK family members (p38 kinase, JNK, and ERK1/2) show different sensitivities to SB202190 and SB203580. Low concentrations (up to 10 μM) selectively inhibit p38 kinase; at 30 μM, both p38 and JNK are inhibited, whereas ERK1/2 is not affected unless higher concentrations are used(35). We therefore tested both concentrations of SB202190. As shown in Fig. 8C and D, a significant decrease was observed in HIRF1/hCAF1 (10-fold) and BTG1 (4-fold)

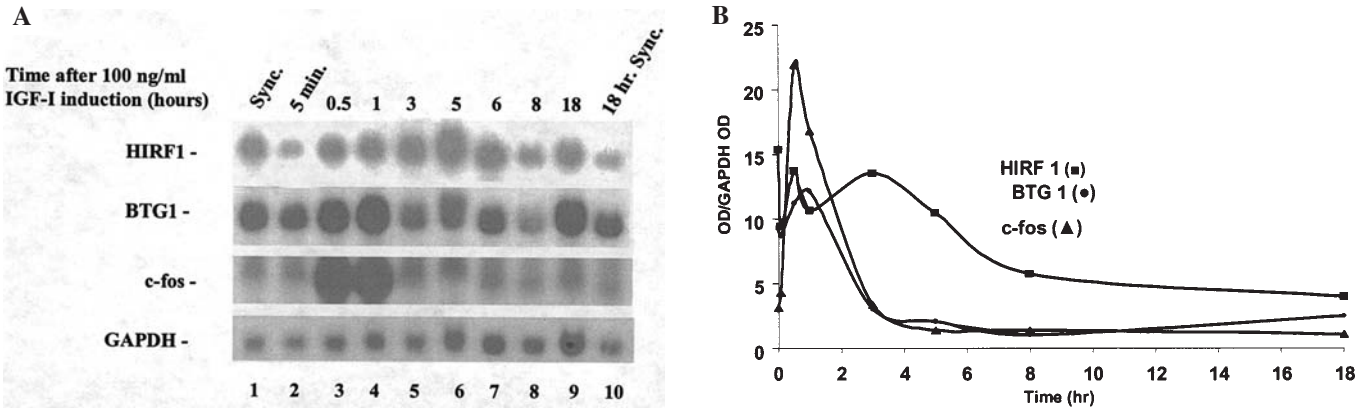

Figure 7. IGF-I induced differential expression of HIRF1/hCAF1, BTG1, and c-fos genes. (A) Total RNA (20 μg) from synchronized MCF-7 cells induced with 100 ng/ml of IGF-I and collected at different times after induction: 0 min, 5 min, 30 min, 1, 3, 5, 8, and 18 h (lanes 1-9) as well as 18 h of extended synchronization (lane 10) were analyzed by Northern analysis using a labeled HIRF1, BTG1, and c-fos probes. The membrane was re-hybridized with labeled GAPDH probe and is shown on the bottom panel. (B) Quantitative representation of the mRNA levels of HIRF1/hCAF1, BTG1, and c-fos after GAPDH correction. HIRF1/hCAF1 is represented by solid line and square points, BTG1 and c-fos are represented by line with circular points, and triangle points respectively.

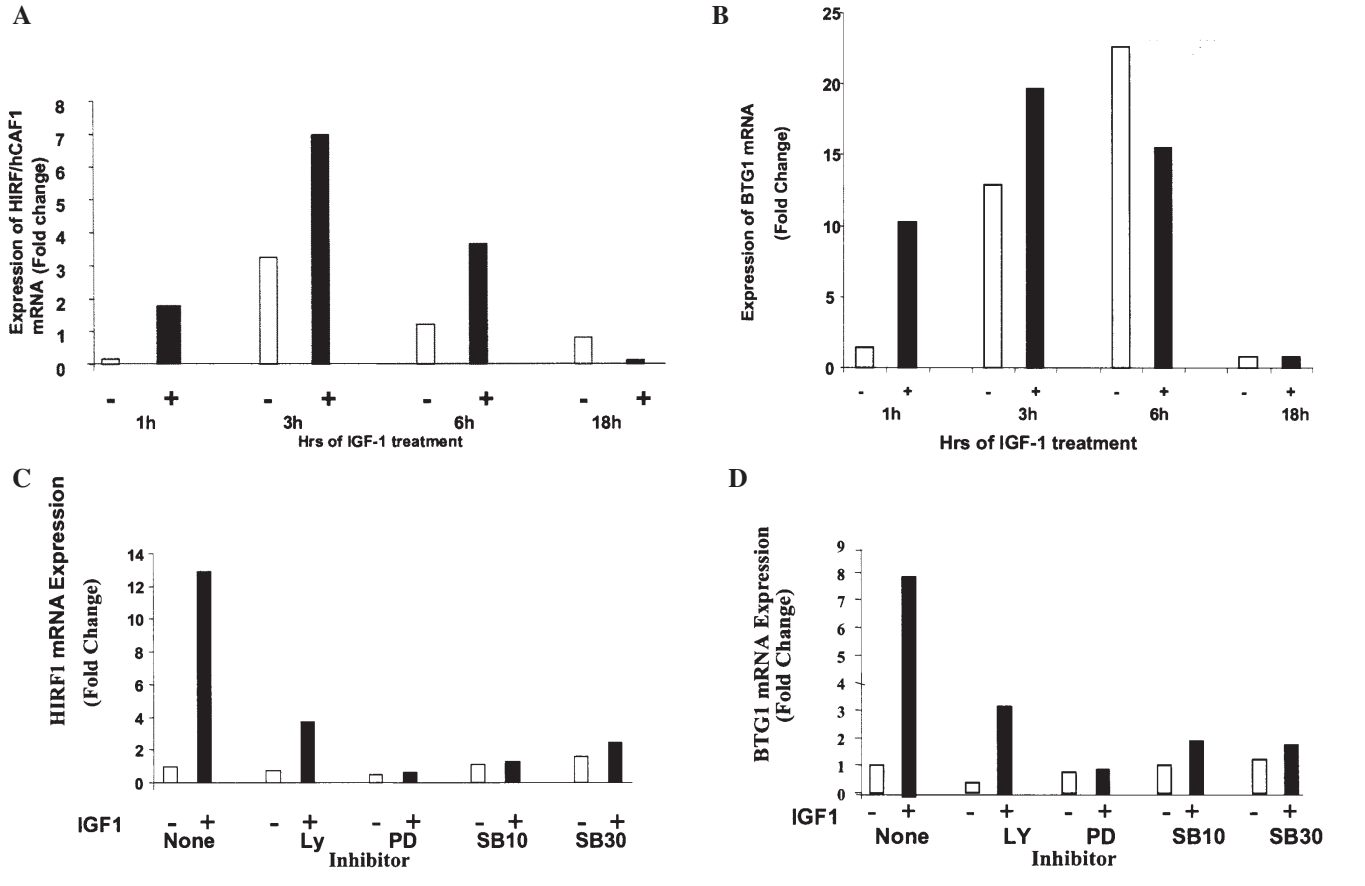

Figure 8. HIRF1/hCAF1 and BTG1 mRNA levels are elevated in MCF-7 cells on stimulation with IGF-1 (50 ng/ml). (A) Determination of HIRF1/hCAF1 mRNA by quantitative RT-PCR from IGF-1 (+)-induced and synchronized control (-) cells compared at 1, 3, 6 and 18 h. (B) Measurements of BTG1 mRNA levels from induced and synchronized control cells at various time points (C). Effect of MAPK and PI3K inhibitors on HIRF1/hCAF1 mRNA: overnight synchronized cells were treated with 50 μM Ly294002, 30 μM PD098059 or SB202190 (10 μM and 30 μM) for 1 h. Fifty μg/ml of IGF-1 was added and incubated for 3 h. Total RNA was extracted from both control (-) without IGF-1 and (+) IGF-1 treated samples. Quantitative RT-PCR for HIRF1/hCAF1 mRNA was performed as outlined before (n=2 at each time point). (D) Effects of Ly294002, PD98050 or SB202190 on BTG1 mRNA expression in MCF-7 cells: BTG1 mRNA expression in synchronized controls (-) and induced cells (+) in presence of 50 μM Ly294002, 30 μM PD98050, 10 μM or 30 μM SB20190. The treatment of cells is the same as outlined in (C) (n=2 at each time point).

mRNA. The inhibition data suggest that the MAPK (both p38 and ERK1/2) pathways are more responsive to the IGF-I-induced up-regulation of HIRF1/hCAF and BTG1 mRNA, compared to the PI3 kinase pathway.

Discussion

In recent years, many reports have been published on the physiological effects of IGF-I as a mitogen for the growth

and proliferation of many different cell types *in vitro* (10,36). The effectiveness of IGF-I as a mitogen for the estrogen receptor positive human breast cancer derived cell line, MCF-7, is well documented (37,38). However, the molecular mechanisms for IGF-I-mediated differential gene expression in breast cancer cells have not been fully characterized. In the present study, we investigated the role of IGF-I on differential gene expression in MCF-7 breast cancer epithelial cells. Our study revealed several differentially expressed genes and, in particular, the mechanisms of IGF-I-induced up-regulation of HIRF1/hCAF and BTG1 genes.

Our results have shown a concomitant increase in level of HIRF1/hCAF1, BTG1 and c-fos mRNA within 5 min of induction by IGF-I. HIRF1, BTG1 and c-fos showed interesting expression patterns on IGF-I induction. After reaching a maximum at 3 h, the transcript level of HIRF1 dropped to below the initial expression level. In contrast, Northern blots of BTG1 and c-fos showed maximal expression at 1 h. Using quantitative RT-PCR analysis, BTG1 and HIRF1/hCAF1 showed maximal expression after 3 h of IGF-I treatment. In HeLa cells, a similar expression pattern was observed for 2 clusters of genes on DTT treatment; and maximal expression was seen at 2 h (39). The rapid and transient induction of these genes by IGF-I could occur through an early response gene expression mechanism. The transcription factor, c-fos, a component of the AP1 transcription complex, is an immediate early gene product.

Insulin-like growth factor (IGF-I) has been shown to play an important role in the survival and proliferation of many tissues, especially neoplastic tissues. Importantly, IGF-I is a potent mitogen for breast cancer cells *in vitro* and *in vivo* (3,40). IGF-I is known to mediate proliferative effects in MCF-7 cells through the activation of both PI3K and MAPK pathways (10,34). Our observation that IGF-I-mediated induction of HIRF1/hCAF1 is abrogated (Fig. 8C) in the presence of 10 μM SB20190 and 30 μM PD98059 points to the involvement of MAPK (specifically p38 and ERK1/2 kinase) pathways. Interestingly, the PI3 kinase inhibitor (LY294002) had less effect on the IGF-I induction of both HIRF1/hCAF and BTG1 mRNA levels, suggesting greater dependence on the MAPK pathways.

The HIRF1/hCAF and BTG1 early response pattern is similar to that of the growth factor-induced c-fos/AP1 proto-oncogene stimulation. AP1, a dimeric transcription factor, is a nodal point for determining cell fate during growth factor stimulation of different cell types. AP1 consists of various combinations of fos and jun family members that dimerize via the leucine zipper domain and bind to specific target sequences (TRE: tetradecanoyl phorbol acetate-responsive element) (41). TREs are found in the promoters of a large number of genes, including cell cycle regulators such as cyclin D1, VEGF and TGF-ß. Expression of c-fos and jun has been widely observed to be temporally regulated in response to diverse stimuli. Consequently, the AP1 dimerization pattern changes with different environmental cues resulting in AP1 transcription activation of target genes (42,43). Serum stimulation of quiescent fibroblasts results in a dramatic (50-fold) increase in mRNA level by 5 min of induction, and declines to basal levels within 30 min (44). The Ras-dependent Raf-MEK-MAPK cascade is the key pathway transmitting signals from growth factors to the nucleus. MAPK has been shown to control c-fos though SRE (serum responsive element) in a transient manner. However, serum and growth factors are required to be present for 8-10 h for sustained activation of MAPK to ensure cell cycle re-entry.

Our data suggest that BTG1, through its association with HIRF1/hCAF, may participate, either directly or indirectly, in the transcriptional regulation of genes involved in the control of the cell cycle. B-cell translocation gene 1 (BTG1) is a member of a new family of putative antiproliferative factors, which are characterized by their rapid but transient expression in response to factors that induce growth arrest and subsequent differentiation (45). The BTG1 gene locus has been shown to be involved in a t(8;12)(q24;q22) chromosomal translocation in a case of B-cell chronic lymphocytic leukemia. Rouault *et al* (26) showed that the full-length cDNA isolated from a lymphoblastoid cell line contains an open reading frame of 171 amino acids. BTG1 expression is maximal in the G0/G1 phases of the cell cycle and is down-regulated when cells progress throughout G1. The BTG1 open reading frame is 60% homologous to PC3, an immediate early gene induced by nerve growth factor in rat PC12 cells. Sequence and Northern blot analyses indicate that BTG1 and PC3 are not cognate genes (46). The BTG family of anti-proliferative gene products includes PC3/TIS21/BTG2, BTG1, Tob, Tob2, Ana/BTG3, PC3k and others. Every member of this family has a potential to regulate cell growth (27).

The molecular function of PC3 (TIS21/BTG2) is still unknown, but its ability to modulate cyclin D1 transcription, or to synergize with the transcription factor Hoxb9, suggests that it behaves as a transcriptional co-regulator (47). Bogdan *et al* (48) were the first to demonstrate that hCAF1 interacts with BTG1 and influences cell proliferation and differentiation. The authors used the yeast 'two-hybrid' system to screen for interacting protein partners and identified human carbon catabolite repressor protein (CCR4)-associative factor 1 (hCAF1), a homologue of mouse CAF1 (mCAF1) and *Saccharomyces cerevisiae* yCAF1/POP2. Collectively, these results indicate that formation of the hCAF1/BTG1 complex is driven by phosphorylation at BTG1 (Ser159) and implicates this complex in the signaling events of cell division that lead to changes in cellular proliferation associated with cell-cell contact.

Up-regulation of BGT1 by IGF-I seems paradoxical, since BTG1 is a member of an antiproliferative gene family that includes Tob1, BTG1, PC3/TIS21/BTG2, PC3B, ANA/BTG3, and Tob2. These proteins participate in transcriptional regulation of several genes and regulate cell growth and differentiation. Exogenous overexpression of the family of these proteins suppresses cell proliferation. Among the BTG/Tob family members, BTG2/TIS21/PC3 proteins have been reported to have short lives and to be degraded by the proteasome. Kawamura-Tsuzuku *et al* (49) showed that Tob is a nuclear protein that is imported into the nucleus through a nuclear localization signal (NLS)-mediated mechanism. Mutation in the NLS sequence of Tob affects its nuclear localization and impairs antiproliferative activity. Additionally, Tob contains a nuclear export signal (NES). In oncogenic ErbB2-transformed cells, nuclear export of Tob is facilitated by an NES-mediated mechanism, resulting in a decrease of

its antiproliferative activity. These results indicate that regulation of the nuclear localization of Tob is important for its anti-proliferative activity. It is highly likely that IGF-I treatment results in the export of BTG1 from the nucleus.

We speculate that IGF-I mediated increase in BTG1 is an early response to IGF-I inducing cell differentiation but not proliferation. As evident from our studies, when cells begin to progress to G1/S phase and proliferate, the levels of BTG1 decrease significantly.

Prevot *et al* (50) examined the physical and functional relationships between the BTG proteins, and their partners, hCAF1 and hPOP2, were investigated to find out how these interactions affect cellular processes and, in particular, transcription regulation. Their data suggests involvement in estrogen receptor α (ERα)-mediated transcription regulation. BTG1 and BTG2 interact with CAF1 via a CCR4-like complex, which can play either positive or negative roles in regulating the ERα function. Furthermore, their data indicate that two LXXLL motifs, referred to as nuclear receptor boxes, present in both BTG1 and BTG2, are involved in the regulation of ERα-mediated activation.

BTG1 may also play an important role in angiogenesis (51). BTG1 mRNA was abundantly expressed in quiescent endothelial cells. Addition of serum and angiogenic growth factors decreased BTG1 mRNA levels in endothelial cells. In contrast, BTG1 mRNA was up-regulated in tube-forming endothelial cells on Matrigel. Inhibition of endogenous BTG1 by overexpression of antisense BTG1 resulted in inhibited network formation and overexpression of sense BTG1 augmented tube formation in these cell lines. BTG1-over-expressing endothelial cells displayed increased cell migration.

It is possible that IGF-I treatment inhibits BTG1 degradation and increases its mRNA stability. By modulation of HIRF1/hCAF1 and BTG1 levels at different times, IGF-I may control transcription and mRNA degradation during different phases of MCF-7 induction. There is evidence that supports the breakdown of BTG1 and related proteins by the ubiquitin-proteosome system. Sasajima *et al* (52) reported that BTG1, Tob, and Tob2 proteins, as well as BTG2 protein, are degraded by the ubiquitin-proteasome system; the degradation of Tob protein in HeLa cells and the degradation of BTG1, BTG2, Tob and Tob2 proteins transiently expressed in HEK293 cells were inhibited by treatment with proteasome-specific inhibitors.

The B-cell translocation gene family (BTG1, BTG2, BTG3, and Tob1) are Arf-responsive genes, in a p53-independent manner. Activation of these genes resulted in inhibition of cell proliferation in primary mouse embryo fibroblasts expressing or lacking functional p53. The tumor suppressor p19(Arf) [p14(ARF) in humans], encoded by the Ink4a/Arf locus, is mutated, deleted, or silenced in many forms of cancer. p19(Arf) induces growth arrest by antagonizing the activity of the p53-negative regulator, Mdm2, thereby inducing a p53 transcriptional response (53).

Antiproliferative- or apoptosis-related genes encoding BTG1, BTG2, and granzyme A were also found to be transcriptionally up-regulated by glucocorticoids (54). BTG1 is highly expressed in androgen-dependent prostate cancer cells (LNCaP-FGC) compared with androgen-independent prostate cancer cells (LNCaP-LNO) (55).

The CCR4-NOT-CAF1 complex has been shown to be a global regulator of transcription in yeast. CCR4-CAF1 up-regulates the transcription of ADHII, genes for non-fermentative growth and cell wall integrity. mCAF1 can replace yCAF1 in its role in transcription regulation. The role of hCAF1 has been postulated to be similar to its orthologs in yeast and mouse. Regulation at a global level affects transcriptional responses of a multitude of functionally unrelated genes and permits cells to adjust for environmental variations such as changes of nutrients or growth factors and cellular differentiation. In yeast, the CCR4-NOT-CAF1 complex appears to act as a pivotal point of signaling pathways towards TBP/TFIID (TATA binding protein) functions; and might be one of several factors that contribute to global gene regulation by modulation of TBP activity. Badarinarayana *et al* (15) have observed a dose-specific functional interaction between CCR4-NOT proteins with TBP and its associated factors. The complex is also known to be a negative regulator of transcription by limiting the access of the TBP binding protein. It has been hypothesized that CCR4-NOT proteins could sequester some components of TFIID and/or SAGA and thereby affect TBP function at certain promoters.

In conclusion, we propose that the cloned HIRF1 gene from estrogen receptor-positive breast cancer cells is identical to the previously cloned BTG1 binding factor 1 or hCAF1 gene. HIRF1/hCAF1 is located at the short arm of the human chromosome 8p21.3-p22 anti-oncogene segment. Differential expression of closely related HIRF1 and BTG1 genes as well as hCAF1/BTG1 complex formation could be highly controlled by IGF-I to regulate signaling events leading to changes in cell differentiation and/or proliferation.

Acknowledgments

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