Autoregulation of Co-Chaperone BAG3 Gene Transcription

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ABSTRACT

The Bcl-2-associated athanogene, BAG, protein family through their BAG domain associates with the heat shock protein 70 (HSP-70) and modulates its chaperone activity. One member of this family, BAG3, appears to play an important role in protein homeostasis, as its expression promotes cell survival. Expression of BAG3 is enhanced by a variety of stress-inducing agents. Here we describe a role for BAG3 to modulate transcription of its own promoter through a positive feedback loop involving its 5′-UTR sequence. Activation of the BAG3 promoter is mediated by the BAG domain and is independent of BAG3 association with the UTR sequence. Autoactivation of the BAG3 gene is observed in several cultures of human glial cells including gliomas, but not in several other non-glial cell lines such as He La and others. Results from cell fractionation and immunocytochemistry showed BAG3 in the cytoplasm as well as the nuclei of glial cells. These observations suggest that BAG3 gene expression is controlled by its own product and that this may be critical for the biological activity of BAG3 in some cell types. J. Cell. Biochem. 108: 1117–1124, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: BAG3; 5′-UTR; POSITIVE FEEDBACK; TRANSCRIPTION REGULATION; Bcl-2 REGULATORY PROTEIN

The co-chaperne protein BAG3 belongs to the evolutionarily conserved Bcl-2-associated AthanoGene (BAG) family of proteins that were originally isolated based on their ability to interact with the anti-apoptotic protein Bcl-2 [Takayama et al., 1995; Lee et al., 1999; Antoku et al., 2001]. Later studies revealed the interaction of BAG3 via its BAG domain, which is located in the C-terminus of the protein, with heat shock protein 70 (Hsp70) and controlling the chaperone activity of Hsp70 [Takayama et al., 1997, 1999]. At the N-terminus, a unique region called the WW domain, which is predicted to bind to proline-rich motifs, differentiates BAG3 from the other members of the BAG3 family [Einbond and Sudol, 1996] A PXXP proline-rich sequence located upstream of the BAG region associates with the SH3 domain of the phospholipase-C-gamma [Doong et al., 2000].

BAG3 has been reported to possess remarkable anti-apoptotic activity by enhancing Bcl-2 activity and promoting survival of normal and neoplastic leukocytes upon oxidative stress conditions [Lee et al., 1999; Bonelli et al., 2004]. Earlier studies showed that BAG3 is able to regulate replication of HSV and modulate transcription of the HIV-1 genome [Rosati et al., 2007; Kyratsous and Silverstein, 2008]. Stress induced upon viral infections can also downregulate BAG3 expression as shown by the ability of JCV early protein to decrease transcription of BAG3 in glial cells [Basile et al., 2009]. Furthermore, BAG3 is involved in the modulation of motility

and adhesion of epithelial cancer cells and regulation of Rac small GTPase activity [Iwasaki et al., 2007]. Recently, a novel role has been ascribed to BAG3 in controlling the turnover of misfolded protein by stimulating macroautophagy [Carra et al., 2008a,b].

BAG3 is expressed at different levels in most tissues, being preferentially overexpressed in skeletal and cardiac muscle [Lee et al., 1999]. BAG3 expression is induced at the transcriptional level by a variety of stimuli, including exposure to heavy metals, heat shock factor-1 and proteosome inhibitors [Pagliuca et al., 2003; Franceschelli et al., 2008; Du et al., 2009]. Earlier studies have shown upregulation of BAG3 upon treatment with FGF2 through stimulation of the transcription factor, Egr-1 [Gentilella et al., 2008]. Here we demonstrate positive autoregulation of BAG3 in glial cells via a region of the BAG3 gene spanning the 5′ UTR.

RESULTS

AUTOREGULATION OF BAG3 PROMOTER

As the delicate balance of BAG3 levels in cells may play an important role in cell response to stress, we sought to investigate the impact of BAG3 on transcription of its own promoter. A reporter plasmid containing a DNA fragment of the BAG3 gene spanning -831 to $+306$ with the transcription start site at $+1$, was introduced into the human glioma cell line, U-87 MG, in the absence and

presence of BAG3 expression plasmid. Results from this experiment showed that an increased level of BAG3 stimulates transcription of the BAG3 promoter and has no effect on the promoter activity of Id-1, which was used as a control in this study (Fig. 1A).

Autoactivation of the BAG3 promoter was also observed in another glioma cell line, T98G, as increasing levels of BAG3 progressively enhanced BAG3 promoter activity (Fig. 1B). Similarly, BAG3 stimulated transcription of its own promoter in primary cultures of human fetal glial cells suggesting that the observed effect is not restricted to tumor cells (Fig. 1C). However, in other cells including HeLa and MCF-7, BAG3 had no impact on the transcriptional activity of its own promoter (data not shown), implying that autoregulation of BAG3 may not be a universal event in all cell types.

BAG DOMAIN IS ACCOUNTABLE FOR BAG3 PROMOTER TRANSACTIVATION

To identify the region(s) within the BAG3 protein that stimulates promoter activity, truncated cDNAs encoding amino acid residues 1–420 and 421–576 were cloned into a pcDNA6-Myc-His expression vector and their activity upon the BAG3 promoter was evaluated by transfection assay. Results of this experiment showed that expression of the BAG3 cDNA encompassing residues 421–576 that contain the BAG domain (residues 421–498) stimulates the activity of BAG3 promoter. Removal of 78 amino acid residues spanning aa 499–576 had no impact on the ability of 421–576 to stimulate the BAG3 promoter (data not shown) Under similar conditions, production of truncated BAG3 spanning residues 1–420 had no significant effect on the BAG3 promoter (Fig. 2B). Results from Western blot using anti-His tag antibody verified production of mutant BAG3 in the cells (Fig. 2B). Again, similar results were obtained with other glial cell lines (data not shown). Examination of the level of the luciferase transcript by Northern blot established that the observed induction of luciferase activity is due to increased level of luciferase transcript as a result of activation of BAG3 promoter (Fig. 2C). It was also observed that only Δ421–578 but not Δ1–420 of BAG3 is able to increase the level of luciferase RNA in the cells. To further establish the involvement of the BAG domain in the activation of the BAG3 promoter, recombinant plasmids expressing two and three copies of the region spanning 421–576 were created and after verification of their production in cells by Western blot (Fig. 3A), their effect on the BAG3 promoter was evaluated in glial cells. As seen in Figure 3A, the level of BAG3 promoter activity was noticeably increased in cells expressing two copies (19.0-fold) and three copies (15.3-fold) of the BAG domain. The reduced level of luciferase activity in cells expressing three tandem copies of the BAG domain in comparison with that for the cells expressing two copies of BAG domain suggests that increasing the number of BAG domains does not proportionally stimulate the BAG3 promoter. It was also noted that BAG5, which contains four divergent copies of the BAG domain, has no ability to stimulate BAG3 promoter activity (Fig. 3B). Altogether, these observations ascribe a role for the BAG domain of BAG3 in upregulation of the BAG3 promoter.
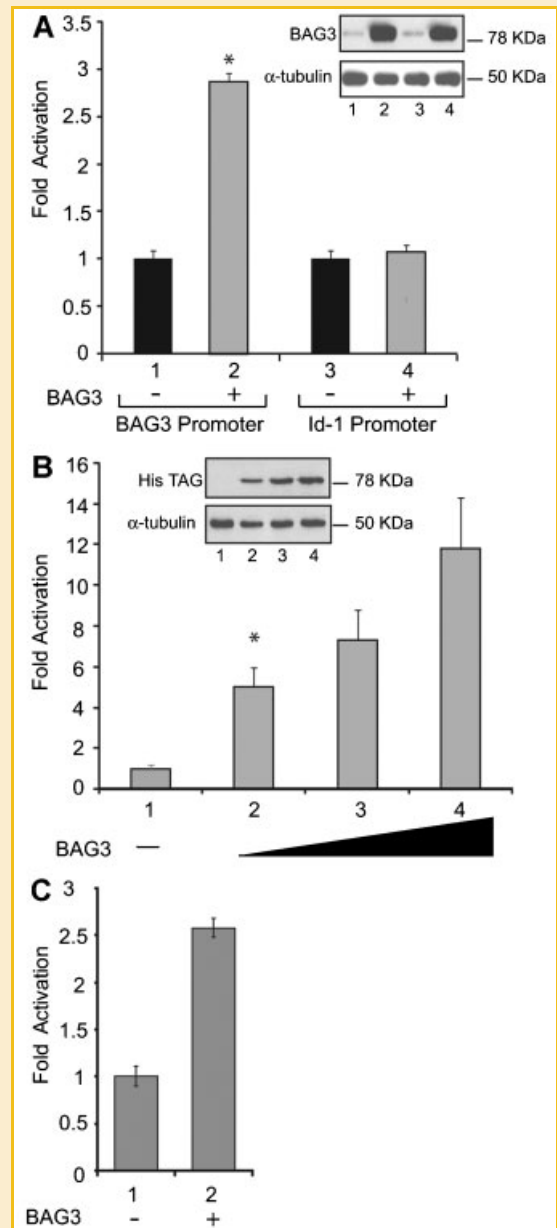

Fig. 1. Activation of the BAG3 promoter by BAG3 protein overexpression. A: Luciferase assay in U-87 MG glioblastoma cells using reporter plasmid containing the BAG3 promoter (−831 to +306) DNA sequence (left bars) or Id-1 promoter (−2114/+95) sequence (right bars) upstream of the luciferase gene. Cells were co-transfected with empty vector or BAG3 full-length cDNA plus TK-Renilla luciferase as normalization control. Twenty-four hours after transfection, cells were harvested and firefly-luciferase and constitutively active thymidine kinase-Renilla luciferase activities were assayed. The data are expressed as fold effect from three independent experiments where luciferase activity was normalized with Renilla expression. B: BAG3 cDNA dose-dependent response of the BAG3 promoter −831 to +306. Increasing amounts of BAG3 full-length cDNA were co-transfected with BAG3 promoter-luciferase in T98G cells as depicted. Firefly-Renilla luciferase assay was carried out and analyzed as in Panel A. BAG3 full-length protein overexpression was evaluated by Western blot; α-tubulin was used as loading control. C: Luciferase assay in primary human fetal astrocytes. Primary human fetal astrocytes were transfected with BAG3 promoter DNA plasmid and luciferase assay was performed according to the method described in Panel A. The asterisks represents $P < 0.01$.
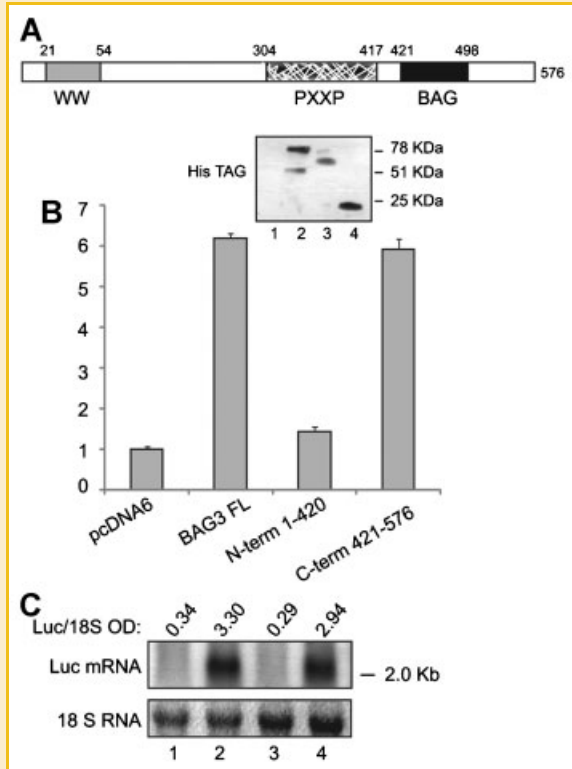
Fig. 2. Identification of BAG3 protein region required for BAG3 promoter activation. A: Schematic representation of BAG3 protein domains. B: T98G cells were co-transfected with the BAG3 promoter sequence −831 to +306. Firefly–luciferase plus empty vector (lane 1) BAG3 FL (lane 2) DC 1–420 (lane 3) or C-term 421–576 (lane 4). Luciferase assay was carried out 24 h after transfection as described above. Ten micrograms of total protein lysates was analyzed by Western blot and hybridized with a polyclonal anti-His antibody raised against the 6× Histidine tag present at the C terminus of recombinant protein. C: T98G cells were transfected as in Panel B. Total RNA was purified and 12 μg were analyzed by Northern blot. The filter was hybridized with firefly–luciferase probe. 18S ribosomal RNA was used as loading normalization control.

Fig. 3. BAG3–BAG domain activates BAG3 promoter (A) Luciferase assay in T98G cells co-transfected with BAG3 promoter −831/+306 + empty vector (lane 1) or 1× (lane 2) 2× (lane 3) and 3× of C–terminus (421–576) tandem repeats (lane 4). Overexpression of transfected cDNAs was assayed in the same lysates by immunoblotting with anti-His polyclonal antibody. B: Luciferase assay in T98G glioblastoma cells co-transfected with equal amounts of the BAG3 promoter (−831/+306) and empty vector (left), BAG3 FL cDNA (middle) or BAG5 cDNA (right) plasmids. Fold activation was evaluated from three independent experiments, after normalization with Renilla activity, considering empty vector as basal luciferase level.

FUNCTIONAL ANALYSIS OF THE cis-REGULATORY ELEMENTS IN THE BAG3 PROMOTER

To identify the region within the BAG3 promoter that is responsible for transactivation by the BAG domain, a series of BAG3 promoter 5′-deletion mutants were used in transfection assays in the absence and presence of a BAG3 expression plasmid. As shown in Figure 4A, removal of the region between −831 and −205 had no significant impact on the level of its transcriptional activation by BAG3. Deletion of the sequence between −205 and −26, which had a significant effect on the basal promoter activity, elevated the level of responsiveness to BAG3. Removal of the DNA sequence corresponding to the 5′-UTR from +157 to +306 reduced the level of transactivation of the promoter. Deletion of the entire 5′-UTR DNA sequence (+1 to +306) completely abrogated the ability of the promoter to respond to BAG3. This observation suggests that the BAG responsive element of the BAG3 promoter resides within the 5′-UTR of the gene.

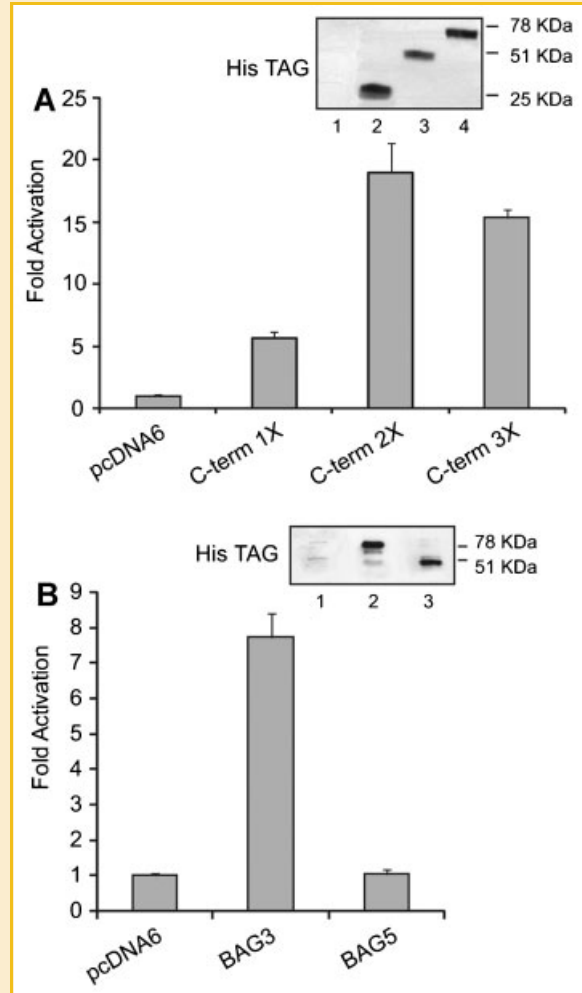In an alternative approach, the level of endogenous BAG3 was reduced upon transduction of the cells with adenovirus expressing BAG3-targeted siRNA and the activity of the full-length BAG3 promoter and the promoter with the 5′-UTR deletion was determined by luciferase assay. As shown in Figure 4B, a decrease in the level of BAG3 protein by Ad-siBAG3 correlates with the decrease in the full-length, but not ΔUTR, promoter. These observations are consistent with promoter deletion observations and establish the 5′-UTR DNA sequence of BAG3 as a target for BAG3 activation.

BAG DOMAIN OVEREXPRESSION REGULATES ENDOGENOUS BAG3 PROTEIN AND RNA LEVELS

In the next series of experiments we focused our attention on the response of the endogenous BAG3 gene to activation by BAG domain. To address this question, T98G cells were transfected with
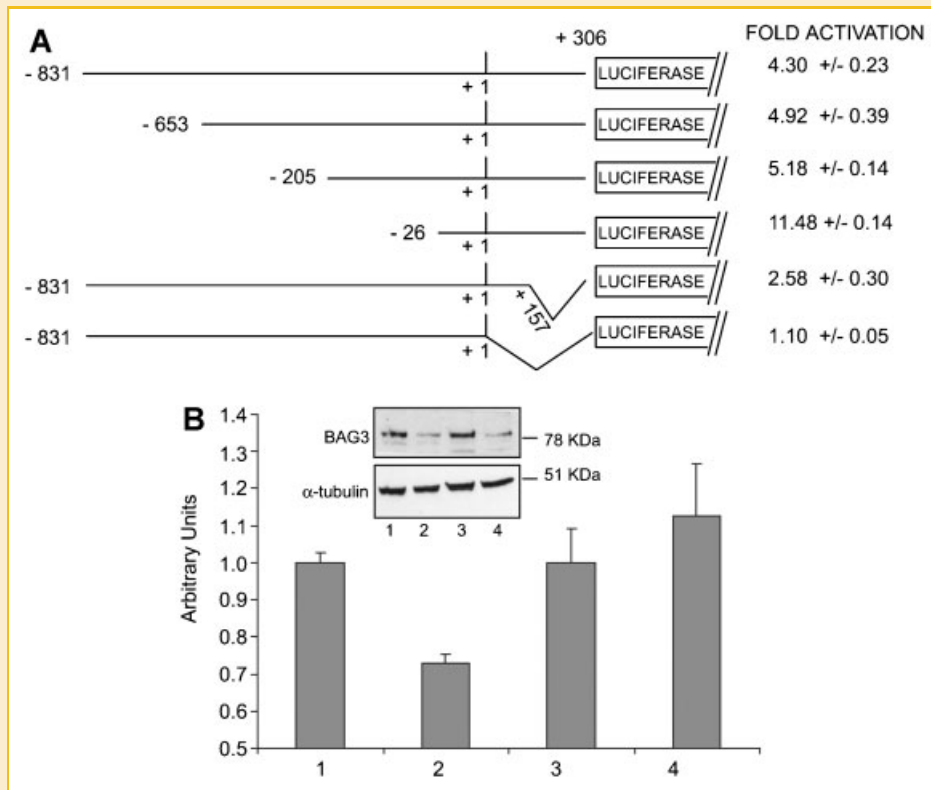
Fig. 4. Identification of BAG3 promoter region responsive to BAG3 full-length protein. A: Promoter-reporter activity of sequential deletions of the BAG3 promoter. At left are the various regions cloned into the pGL3 basic plasmid. The numbers indicate the positions relative to the transcription start site at +1. Fold activation was determined from three independent experiments, after normalization by Renilla activity, by comparing BAG3 FL with empty vector transfected cells. B: T98G cells were infected at 20 m.o.i. with Adenovirus-Null (lanes 1 and 3) or Adenovirus-expressing BAG3-targeting siRNA (lanes 2 and 4) for 24 h. Cells were then transfected with equal amounts of BAG3 promoter-Luciferase −831/+306 (lanes 1 and 2) or −831/+1 (lanes 3 and 4) in both infected conditions. Twenty-four hours post-transfection cells were harvested and analyzed by luciferase assay as described above. Luciferase activity was present in arbitrary units of 1–20. Ten micrograms of whole cell lysate were checked by Western blot for BAG3 expression.

an expression plasmid that produces only one or two copies of the BAG3 C-terminal region 421–576, and at various times cell extracts were prepared for analysis by Western blot using anti-BAG3 antibody. Results from this experiment showed a gradual increase in the level of 80 kDa BAG3 protein level during the course of transfection upon expression of one copy of BAG (Fig. 5A) or two copies of BAG (Fig. 5B) domains. Consistent with the results from the luciferase assay, expression of the double BAG domain has higher stimulating impact than that seen with a single BAG domain. The level of truncated peptide corresponding to single and double C-terminal regions was also detected by Western blot. The level of α-tubulin, which remained constant, served as a control for the experiment (analysis shown in Fig. 4, lower panels). Evaluation of the BAG3 endogenous transcript by qRT-PCR from the C-terminus 2× transfected cells also produced a progressive increase over time, accordingly, with the observed data at the protein level (Fig. 5C).

SUBCELLULAR LOCALIZATION OF BAG3

In light of earlier studies describing BAG3 as a cytoplasmic protein and the ability of BAG to stimulate gene transcription, we decided to examine the subcellular localization of BAG3 in cells over-expressing BAG3. To this end, nuclear and cytoplasmic fractionation from untransfected and cells transfected with BAG3

was prepared and examined by Western blot. As shown in Figure 6A, nuclear extracts from untransfected cells showed BAG3 predominantly in the cytoplasm, whereas in transfected cells, higher levels of BAG3 were detected in both cytoplasm and nuclei of the cells. The purity of each fraction was confirmed by sole detection of α-lamin A/C and α-tubulin in nuclei and cytoplasm, respectively.

To further verify the nuclear localization of BAG3, untransfected cells or cells transfected with plasmids expressing full-length or the C-terminus 421–576 of BAG3 were immunolabeled with anti-BAG3 antibody (FITC). As seen in Figure 6B, significant levels of full-length BAG3 or its C-terminus containing BAG domain was detected in the nuclei of transfected cells. These observations establish nuclear appearance of BAG3 in cells overexpressing this protein.

DISCUSSION

In addition to its role in the translation of RNA, the 5′-UTR of eukaryotic genes plays an important role, at the DNA and/or RNA levels, in the control of gene transcription. In this regard, HIV-1 has received much attention due to the ability of its transactivator, Tat, to stimulate transcription initiation and elongation of the viral
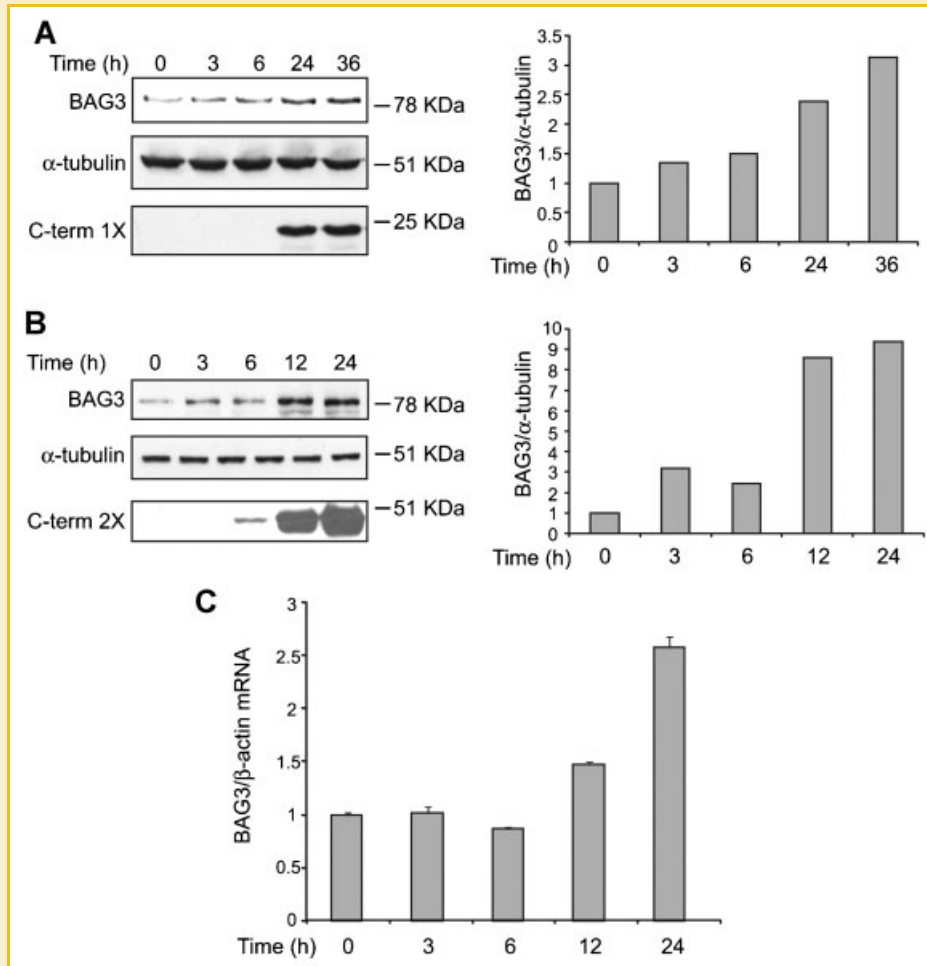
Fig. 5. Induction of endogenous BAG3 by C–terminal BAG in T98G cells. A: Time course of C–terminus 1× overexpression in T98G cells. After transfection, cells were harvested at indicated time points. Forty micrograms of lysate was subjected to Western blot and probed with rabbit polyclonal antibody raised against the full–length BAG3 protein. B: Time course of C–terminus 2× overexpression in T98G cells. The procedure was performed as in Panel A. C: qRT–PCR analysis of RNA in the same samples as in Panel B. The BAG3 transcript was measured and β–Actin mRNA was used as the normalization control.

genome through interaction with the RNA sequences spanning the 5′-UTR and recruitment of other regulatory proteins including pTEF. Transcription of several cellular genes including Pim-1 and Nox-1 is also regulated by the DNA region within the 5′-UTR [Wang et al., 2005; Uittenbogaard et al., 2007].

Among the BAG family of inducible proteins, expression of BAG1 is rapidly enhanced at the translational level, in response to heat shock via an internal ribosomal entry site positioned in the 5′-UTR [Coldwell et al., 2001]. Here we described a novel pathway that affects expression of BAG3 at the transcriptional level using the DNA sequences spanning 5′-UTR. This event, which is initiated by the BAG domain located in the C-terminus of BAG3, results in the enhancement of the gene at the transcriptional level. While the biological importance of this event remains to be elucidated, autoactivation of BAG3 transcription brings a unique capacity for its product to tightly self-regulate its level in response to various physical conditions. The underlying mechanism by which BAG3 stimulates its own transcription through 5′-UTR DNA sequence is not fully uncovered at present. Our attempts using various DNA–protein interactions including band-shift, footprinting, and ChIP assay showed no conclusive evidence for the direct association of BAG3 with the 5′-UTR DNA sequence. Moreover, our results showed that placing the BAG3 5′-UTR between the transcription and translation start sites of a heterologous gene failed to confer BAG3 responsiveness to other heterologous promoters. Thus, it appears that BAG3 indirectly communicates with its own 5′-UTR sequence and that its effect on the promoter may require sequences upstream from the transcription start site, most notably those spanning between −26 and +1 (based on results from promoter deletion studies). It was also noted that the removal of the DNA sequences between −205 and −26 enhanced the level of transactivation of the BAG3 promoter. This observation suggests that the upstream DNA promoter elements and their corresponding factors can positively and negatively regulate the level of BAG3 activity upon its 5′-UTR DNA motif. According to one model, by communicating with DNA-based proteins at the 5′-UTR, BAG3 recruits upstream transcription factors to the −26 to +1 region and hence, stimulates transcriptional activity of the BAG3 minimal promoter.
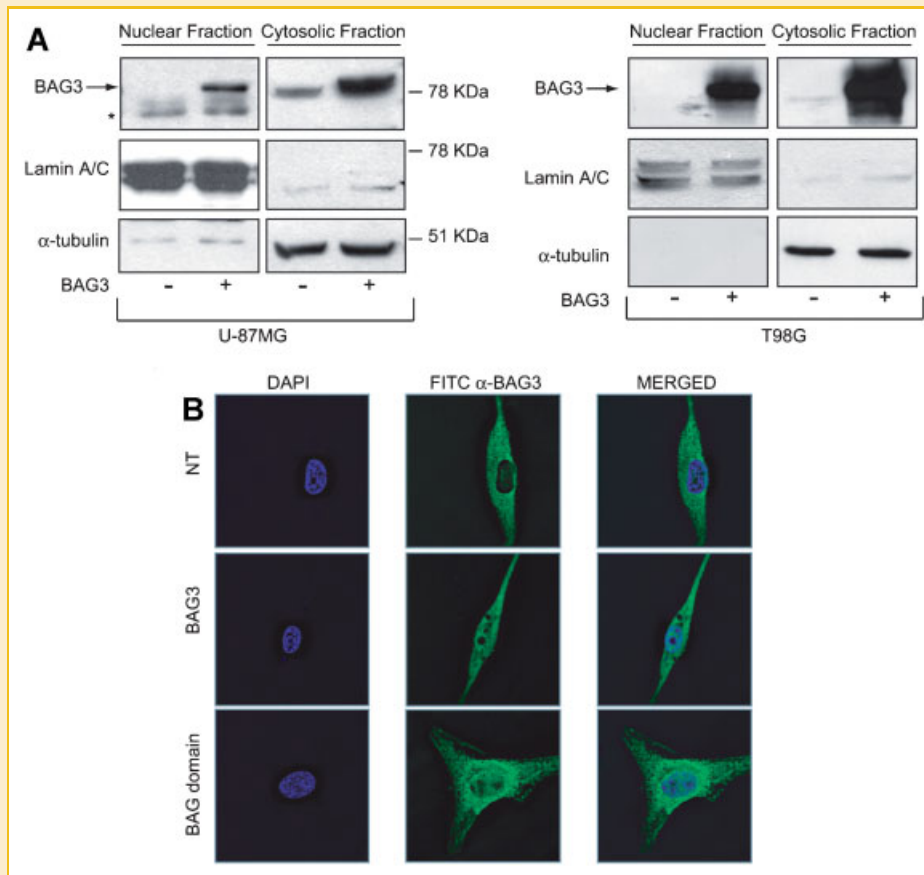
Fig. 6. Subcellular localization of BAG3. A: Untransfected cells and cells (U-87 MG and T98G) transfected with BAG3 FL were harvested after 24 h and nuclear–cytoplasm fractionated as described in Materials and Methods Section. Five and 15 μg of nuclear and cytoplasmic fractions, respectively, were evaluated by Western blot and probed with a rabbit polyclonal antibody against BAG3. The position of BAG3 is shown by an arrow. An asterisk points to a non-specific band. The purity of fractionation was checked by hybridization with anti–Lamin A/C and anti–α–tubulin antibodies. B: U-87 MG cells were transfected as in A, hybridized with polyclonal BAG3 antibody and FITC–labeled secondary anti–rabbit, and microscopically analyzed.

Autoregulation of BAG3 may not be a general event in a variety of cell lines. T98G, U-87 MG, and primary human glial cells, but not HeLa and MCF-7, are responsive to BAG3 and can be activated by its own product, once again implying that this event requires involvement of other cellular factors which are found in glial cells.

Immunomodulation of BAG3 by siRNA in T98G cells showed that endogenous levels of BAG3 are involved in sustaining the basal transcription of BAG3 through the 5'-UTR DNA region (Fig. 4B). On the other hand, the amounts of BAG domains expressed in the cells are important in determining the extent of the positive transcription feedback. While a proportional increase in gene transcription is observed during BAG domain overexpression, no additional increase occurs when the BAG domain is overexpressed over a defined level (Figs. 3A and 5B).

In conclusion, autofeedback appears to be a novel mechanism of BAG3 in glial cells, which may be important in the stress response.

MATERIALS AND METHODS

CELL CULTURE

U-87 MG and T98G human glioma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). Primary human fetal astrocytes were prepared according to the procedures described previously [Basile et al., 2009].

MATERIALS

[α^{32}P]dCTP was obtained from PerkinElmer Life Sciences (Waltham, MA). LipofectAMINE2000 reagent was from Invitrogen, Fugene 6 from Roche (Basel, Switzerland). Bradford Reagent was from BioRad (Hercules, CA). Polyclonal anti-BAG3 antibody was from Alexis Biochemicals (San Diego, CA) and Proteintech Group (Chicago, IL). Polyclonal anti-His tag was from Cell Signaling. Anti-α-tubulin (clone B-5-1-2) was purchased by Sigma–Aldrich (St. Louis, MO). Anti-Lamin A/C was from Cell Signaling. Goat anti-(mouse IgG)-peroxidase conjugate and goat anti-(rabbit IgG)-peroxidase conjugate were from Pierce (Rockford, IL).

CONSTRUCTION OF ADENOVIRAL VECTOR EXPRESSING siRNA AGAINST HUMAN BAG3

For construction of the vector, a commercially available kit from BD Biosciences/Pharmigen (La Jolla, CA), namely BD^{TM} Knockout RNAi

system, was used. The system utilizes the RNA PolIII-dependent human U6 promoter to express a small hairpin RNA (shRNA). The target sequence for h-BAG3 gene was 5′-AAGGUCAGACCAU-CUUGGAA-3′. In order to design shRNA that would ultimately produce siRNA, double stranded DNA strands for this target sequence was chemically synthesized according to the guidelines outlined in the BD Bioscience protocol (PT3739-1). The two DNA strands for h-BAG3 gene were as follows. The top strand was 5′-GATCCGAAGGTTCAGACCATCTTGGAATTCAAGAGATTCCAA-GATGGTCTGAACCTTCTTTTTTGCTAGCG-3′ and the bottom strand was 5′-AATTCGCTAGCAAAAAAGAAGGTTCAGACCATCTTGGAA-TCTCTTGAATTCCAAGATGGTCTGAACCTTCG-3′ (NheI site under-lined). The top and bottom strands were annealed and then cloned into the BamHI and EcoRI sites of RNAi-Ready pSIREN-DNR vector, and recombinant clones containing shRNA coding sequences were isolated on the basis of the presence of the NheI site. The recombinant plasmid was further used to introduce the shRNA coding sequences into adenoviral backbone using Cre and LoxP mediated recombination as per manufacturer's guidelines (BD Bioscience manual PT3674-1). Adenoviral vector thus generated (Adsi-h-BAG3) was further purified on cesium chloride density gradient centrifugation before analyzed for its ability to silence h-BAG3 gene in cultured cells. As a control, an adenoviral vector (Ad Null) was generated using an empty pSIREN-DNR vector.

PROTEIN ANALYSIS

Cell protein extracts were prepared as described previously [Zapata et al., 1998] and quantitated by Bradford Assay (BioRad). Ten to forty micrograms were resuspended in SDS-sample buffer and after treatment at 95°C for 5 min, proteins were size-fractionated by 10% SDS–PAGE, and transferred to nitrocellulose membranes. Blots were stained with Ponceau S to confirm equal loading and transfer of proteins and then reacted with the indicated antibodies. Immuno-blots were developed using appropriate secondary horseradish peroxidase-coupled antibodies and an enhanced chemilumines-cence plus (ECL) kit (Pierce).

TRANSFECTION AND LUCIFERASE ASSAYS

T98G cells were transfected using Fugene 6 (Roche). Cells were plated at a density of 10×10^5 cells/well in a 12-well Plate 24 h prior to transfection. Transfection was performed according to the manufacturer's protocol with 0.75 µg of reporter plasmid, 1.0 µg of pcDNA6Myc-His plasmid and 250 ng of Renilla luciferase control plasmid (Promega) for 24 h. Cell extracts were subsequently prepared and assayed using the Dual Luciferase assay kit (Promega, Madison, WI) as per the manufacturer's instructions. Luciferase activities were normalized to the Renilla control plasmid, and values shown are the mean of three independent experiments

RNA ANALYSIS

Total cellular RNA (1.8×10^5 cells per sample) was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Twelve micrograms of total RNA were resolved on formaldehyde-containing 1.2% agarose gel and transferred to Hybond-N^{TM} nylon membranes (GE Healthcare) and cross-linked by UV-irradiation. A 357 bp DNA fragment corresponding to the Firefly

Luciferase transcript 1105–1462 was radiolabeled with [α^{32}P]dCTP and used as a probe. The filter was pre-hybridized for 1 h at 48°C in UltraHyb solution (Ambion) and hybridized for 20 h at 48°C with cDNA probe for luciferase (10^6 cpm/ml). The blot was then rinsed twice for 5 min with 2× SSC, 0.1% SDS at 50°C and washed twice with 0.2× SSC, 0.1% SDS at 50°C for 20 min. The filter was exposed at −80°C to Fuji XAR-5 film with Kodak intensifying screen. To verify equivalent RNA loading on Northern blot membrane, the filter was rehybridized with GAPDH cDNA probe.

REAL–TIME PCR

RNA was purified as described above. cDNA synthesis and quantitative real-time PCR was performed as described previously [Gamerdinger et al., 2009].

IMMUNOCYTOCHEMICAL STAINING

U-87 MG cells were grown in 2-well glass chamber slides. Cells were fixed with 4% (w/v) PFA for 10 min at room temperature. After a 0.2% Triton X-100 permeabilization step for 5 min at room temperature, non-specific antibody binding sites were blocked with 10% (v/v) normal goat serum. After an overnight incubation of cells with a 1:400 dilution of polyclonal anti-BAG3 antibody (Proteintech Group) in 2% normal goat serum, cells were incubated with FITC-conjugated anti-rabbit antibody (Pierce). Coverslips were mounted using Vectashield with DAPI (Vector Labs, Burlingame, CA).

STATISTICAL ANALYSIS

All statistical analyses have been done by STDEV and AVERAGE programs.

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