BAG3 Expression in Glioblastoma Cells Promotes Accumulation of Ubiquitinated Clients in an Hsp70-dependent Manner*\textsuperscript{[S]}

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Disposal of damaged proteins and protein aggregates is a prerequisite for the maintenance of cellular homeostasis and impairment of this disposal can lead to a broad range of pathological conditions, most notably in brain-associated disorders including Parkinson and Alzheimer diseases, and cancer. In this respect, the Protein Quality Control (PQC) pathway plays a central role in the clearance of damaged proteins. The Hsc/Hsp70-co-chaperone BAG3 has been described as a new and critical component of the PQC in several cellular contexts. For example, the expression of BAG3 in the rodent brain correlates with the engagement of protein degradation machineries in response to proteotoxic stress. Nevertheless, little is known about the molecular events assisted by BAG3. Here we show that ectopic expression of BAG3 in glioblastoma cells leads to the activation of an HSF1-driven stress response, as attested by transcriptional activation of BAG3 and Hsp70. BAG3 overexpression determines an accumulation of ubiquitinated proteins and this event requires the N-terminal region, WW domain of BAG3 and the association of BAG3 with Hsp70. The ubiquitination mainly occurs on BAG3-client proteins and the inhibition of proteasomal activity results in a further accumulation of ubiquitinated clients. At the cellular level, overexpression of BAG3 in glioblastoma cell lines, but not in non-glial cells, results in a remarkable decrease in colony formation capacity and this effect is reverted when the binding of BAG3 to Hsp70 is impaired. These observations provide the first evidence for an involvement of BAG3 in the ubiquitination and turnover of its partners.

The transcriptional response of the cell to proteotoxic stress (the protein stress response) is induced by a plethora of physical conditions and chemical compounds. The heat shock factor-1 (HSF1)\textsuperscript{2} is considered the master regulator that orchestrates this cellular process (1). Hsp(s) and other stress-activated genes, whose activities help the cell to recover from a proteotoxic stress, contain, in their regulatory regions, the so-called heat shock elements (HSEs), which confer stress regulation to those genes (2). Multiple layers of regulation characterize the activation of HSF1. Upon exposure to stressfull conditions, HSF1 accumulates in the nucleus and, by a homotrimerization process, gains the ability to bind to HSE and to transactivate the target genes (reviewed in Ref. 3).

The stress-inducible molecular chaperone Hsp70 is a target of HSF1, and its expression, in combination with other HSF1-target genes, plays a central role in the “tragic” of damaged proteins either by refolding altered conformations or, as a last resort, by promoting their clearance (4). Indeed, the ability of Hsp70 to sense the folded state of a specific client can be coupled to the ubiquitin proteasome system (UPS) by the co-chaperone CHIP (C terminus of Hsp-70-interacting protein) to promote the degradation of the substrate (5, 6). At the same time, Hsp70 has been shown to play a role in autophagy, the catabolic process characterized by the disposal of aggregated proteins and altered cellular structures, by helping substrate translocation through the lysosomal membrane (7).

The co-chaperone BAG3 has been isolated in an attempt to identify proteins that bind to the ATPase domain of Hsp70, a property that BAG3 shares with the other five members of the BAG family (8). As are other stress-regulated genes, BAG3 is activated by an increasing number of stressful and physiological conditions (9–12) and it has been shown to decrease the folding activity of Hsp70 \textit{in vitro} (13). Proteasome inhibitor treatment, a condition characterized by the accumulation of ubiquitinated proteins, stimulates BAG3 transcription in a HSF1-regulated manner (14, 15, and 16). Recent evidence has shown that BAG3 participates in the protein quality control (PQC) process and it can stimulate the macroautophagic pathway during the aging of the rodent brain (17). Furthermore, BAG3 expression stimulates the disposal of polyglutamine (poly Q)-containing proteins, preventing the formation of aggregates (18).

Although BAG3 is not required for proper development of skeletal muscle, its deficiency results in an impaired ability for the preservation of homeostasis in the adult muscle (19). The interplay between BAG3 and the protein degradation pathways has been proposed to mediate this effect (20).

The control of the protein degradation by the use of proteasome inhibitors was shown to be an effective \textit{in vitro} strategy to reduce the proliferative potential of brain tumors such as glioblastoma multiforme (GBM), the most common and aggressive
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brain tumor (21). Here we show that the ectopic expression of BAG3 in glioblastoma cells activates HSF-1, which in turn stimulates the transcription of target genes involved in the stress response such as Hsp70 and BAG3 itself. Evidently, intracellular accumulation of ubiquitinated proteins contributes to the underlying mechanisms participating in the stress response. Ectopic expression of BAG3, through the interaction with Hsp70 and a functional WW domain, a region located in the N terminus of the protein, results in an accumulation of ubiquitinated partners. This event favors the turnover of BAG3-partners, as suppression of polyubiquitin chain formation or proteasomal inhibition results in further accumulation of ubiquitinated clients. At the cellular level, overexpression of BAG3 in glioblastoma cell lines, but not in non-glial cells, results in a remarkable decrease in the colony formation capacity and this tumor suppressor-like effect is almost entirely reverted when the binding to Hsp70 is impaired.

EXPERIMENTAL PROCEDURES

Cell Culture—T98G and U-87MG human glioblastoma cells and HeLa cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA).

Plasmids and Reagents—Nuclear and Cytoplasmic Extraction Reagent was from Pierce. Eugene 6 and SYBR green qPCR kit were purchased from Roche (Basel, Switzerland). Bradford Reagent was from Bio-Rad. Polyclonal anti-BAG3 antibody was from Alexis Biochemicals (San Diego, CA) and Proteintech Group (Chicago, IL). Polyclonal anti-His Tag and monoclonal anti-Myc Tag (9B11) from Cell Signaling (Danvers, MA). Anti-α-tubulin (clone B-5–1-2) was purchased from Sigma-Aldrich. Anti-Lamin A/C was from Cell Signaling. Polyclonal anti-HSF1 (SPA901), anti-Hsp70 (SPA810) and anti-Hsp27 (SMC161) were purchased from Stressgen-Assay Design (Ann Arbor, MI). Anti-Hsc/Hsp70 (W27) and anti-ubiquitin (P4D1) were from Santa Cruz (Santa Cruz, CA). Anti-PLC-γ antibody was from BD Bioscience (Franklin Lakes, NJ). Protein A/G mix was from Invitrogen. Goat anti-(mouse IgG)-peroxidase conjugate and horseradish peroxidase-coupled anti-

bodies and an enhanced chemiluminescence plus (ECL+) kit (GE Healthcare). Every blot shown represents the average result from 2–4 independent experiments.

Glutathione S-Transferase (GST) Fusion Proteins and GST Pull-down Experiments—GST and GST fusion proteins were expressed in Escherichia coli DH5α cells transformed with pGEX5X1, pGEX5X1-BAG3 wt or pGEX5X1-BAG3 R480A. GST fusion proteins were purified by affinity chromatography with glutathione-Sepharose (Amersham Biosciences Pharma-

cia) beads and protein concentration was estimated on a Coomassie Blue-stained SDS/PAGE gel. Equimolar amounts of different GST and GST fusion protein beads (25 μl) were mixed with 500 μg of protein lysate from T98G cells prepared in binding buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/1 mM EDTA/0.3 mM DTT/0.2% Nonidet P-40/0.5 mM PMSF/1 μg/ml leupeptin/2 μg/ml aprotinin and pepstatin). After an overnight incubation, beads were extensively washed 3–5 times with wash buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/1 mM EDTA/0.3 mM DTT/0.5% Nonidet P-40, and mixture of protease inhibitors as described above). Bound proteins were separated on SDS/10% PAGE and transferred to nitrocellulose membrane.

Luciferase Assay—T98G cells were transfected using Eugene 6 (Roche). Cells were plated at a density of 10 × 105 cells/well in a 12-well plate 24 h prior to transfection. Transfection was performed according to the manufacturer’s protocol with 0.4 μg of reporter plasmid, 0.5 μg of pcDNA6-Myc-His plasmid and 0.1 μg 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 Renilla control plasmid, and values shown are the mean of three independent experiments.

Mutagenesis—Site-directed mutagenesis was performed in plasmid-containing −831/+306-Luciferase of the BAG3 promoter by PCR using the QuikChange II site-directed mutagenesis kit (Strategene, La Jolla, CA) according to the supplier’s protocol. The following nucleotides were used as primers for creating the nucleotide substitutions. HSE-binding site is underlined and the altered nucleotides are in bold: HSE wt, 5′-ACT TCT CTC GAC TGG TGG AGA AGT TGG TAC TAG CCG GCC AGT TGC TAC C-3′; HSE Mut, 5′-ACT TCT CTC GAC TGG TGG AGA AGT TGG TAC TAG CCG GCC AGT TGC TAC C-3′.

Similarly, site-directed mutagenesis was performed on BAG3 pcDNA6 Myc-His plasmid to obtain mutation of the arginine 480 into alanine. Primers used to create nucleotide substitutions. HSE-binding site is underlined and the altered nucleotides are in bold: HSE wt, 5′-ACT TCT CTC GAC TGG TGG AGA AGT TGG TAC TAG CCG GCC AGT TGC TAC C-3′; HSE Mut, 5′-ACT TCT CTC GAC TGG ACC ACT AGT TAG CCG GCC AGT TGC TAC C-3′.

Real-time PCR—Total cellular RNA (1.8 × 105 cells per sample) was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA synthesis and quantitative real-time PCR was performed as described previously (16). The sequence of primers used to detect HspA1 and β-actin cDNAs are below: HspA1_F, 5′-ACT GCC CTG ATC AAG CGC-3′; HspA1_R, 5′-CGG GTT GGT TGT CGG AGT...
AG-3'; Actin_F, 5'-CTA CAA TGA GCT GCG TGT GGC-3';
Actin_R, 5'-CAG GTC CAG ACG CAG GAT GGC-3'.

Co-immunoprecipitation Assays—T98G cells were plated in
60-mm dishes at 6.0 × 10^5 cells. Cells were transfected with 4
μg of plasmids using Fugene 6 (Roche), and 24 h later cells were
lysed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.1%
Na deoxycholate, 2 mM EDTA, 10 mM NaF, 1.0% Nonidet P-40,
protease inhibitor mixture, 2 mM Na3VO4, 1 mM PMSF, NEM 2
mM). Cell lysates were centrifuged and cleared from debris and
α-Myc TAG antibody or control IgG were added to 500 μg of
crude protein extract (750 μg for R480A sample in Fig. 3D and
1 mg in Fig. 3E). After an overnight incubation at 4 °C, protein
A/G-Sepharose beads were added for additional 2 h. Beads were
washed three times with RIPA buffer and then resuspended in
one volume of 2× Laemmli buffer. Co-immunoprecipitated
complexes were resolved on a 7% SDS-PAGE. Co-immunopre-
cipitation in denaturing conditions was performed as described
elsewhere (24). Briefly, the cell pellet was lysed in 0.1 ml of 1%
SDS and incubated 10' at 95 °C. Lysates were subsequently
incubated with 0.1 ml of 10% Triton X-100 and 0.8 ml of lysis
buffer on ice for 30 min. After this step the co-immunoprecipi-
tation was carried out as described above.

Colony Formation and Soft Agar Assays—In colony forma-
tion assay, 24 h post-transfection of the plasmids indicated
expressing blasticidin resistance, the cells were seeded at a den-
sity of 1000/ml on 100-mm dishes. Colonies were allowed to
grow for 15–20 days in regular medium supplemented with
blasticidin (5 μg/ml). Positive colonies (more than 100 cells/
colony) were methylene blue-stained and counted. The results
are expressed as percentage of colonies with respect to empty
vector at 100%. The data were obtained from three different
experiments. Soft agar assay of transfected BAG3 isoforms.
One day after transfection with BAG3 plasmids, cells were har-
vested and mixed with tissue culture medium containing 0.5%
soft agar to result in a final agar concentration of 0.25%. 2 ml of
this cell suspension were immediately plated on 60-mm dishes
covered with 0.5% agar in tissue culture medium. Cells were
cultured at 37 °C with 7% CO2 for 12–14 days and stained with
0.0025% crystal violet for 4h. Three different experiments were
carried out for every BAG3 isofrm.

Statistical Analysis—STDEV and AVERAGE programs were
used for statistical analysis.

RESULTS

Like Hsp70, BAG3 transcriptional activation of BAG3 is a
major hallmark of the stress response, which is activated by
HSF-1. Earlier studies have shown that proteasome inhibitor
MG132 stimulates BAG3 gene transcription by recruiting heat
shock factor 1 (HSF1) and that DNA sequences positioned
within the 5'-UTR of the BAG3 gene are required for this event
(14). This is an interesting observation in light of our recent
results on the auto-activation of the BAG3 promoter and the
importance of the 5'-UTR for this positive regulatory feedback
(22). As a first step to investigate potential involvement of HSF1
in the auto-regulation of BAG3, human glioblastoma cells,
T98G, were transfected with plasmids encoding either full-
length BAG3 or its mutant variant ΔC-(1–420), which lacks the
C terminus of the protein. Examination of the nuclear extracts
from these cells revealed a remarkable increase in the level of
HSF1 in cells overexpressing full-length BAG3 but not mutant
ΔC-(1–420) (Fig. 1A). Similar results were obtained with other
human glioblastoma cell lines, such as U-87MG but not with
cells of non-glial origin including HeLa cells (data not shown).

To investigate involvement of HSF-1 in transcriptional activ-
iation of BAG3, the nucleotide sequence corresponding to the
HSE present in BAG3 5'-UTR was altered in the context of the
firefly luciferase reporter gene construct bearing the BAG3 pro-
moter sequence spanning nucleotides −831 to +306 nucleo-
tides, with respect to transcription start site at +1. As shown by
luciferase activity assay (Fig. 1B), mutation in the HSE desensi-
tized the BAG3 promoter for activation by full-length BAG3,
suggesting that this effect is mediated by HSE-binding nuclear
proteins, including HSF1. In fact, results from band shift studies
verified the interaction of HSF1 with the HSE motif of BAG3
(data not shown).

HSF1 is the master regulator of heat-shock and protein stress
response (1). Activation of HSF1 leads to enhanced tran-
scriptional activity of Hsp70 and the related proteins. To further
investigate the biological impact of BAG3-HSF-1 cross activa-
tion, first we examined the level of Hsp70 transcription in T98G
cells after transfection with plasmids expressing full-length
BAG3 or its mutant variant ΔC-(1–420). Results from RNA
analysis revealed induction of Hsp70 transcription by full-
length BAG3, but not its mutant variant ΔC-(1–420) (Fig. 1C),
suggesting that activation of HSF-1 by BAG3 triggers expres-
sion of the stress protein response.

Interestingly, down-modulation of endogenous BAG3
expression by BAG3 specific siRNA caused more than 40%
decrease in the level of both Hsp70 and Hsp27 expression. Of
note, suppression of BAG3 expression had no major impact on
the level of Hsp40 and α-B crystallin in these cells (data not
shown). Altogether, these observations indicate that endoge-
nous BAG3 sustains the expression of some HSF1 target genes
(Fig. 1D).

The BAG domain has been shown to play a role in the BAG3
autoregulatory feedback as its deletion from the full-length pro-
tein impairs BAG3 transcriptional activation (22). The BAG
domain is also the region of the BAG proteins that retains the
ability to interact with Hsp70 (13, 25).

To test the hypothesis of an involvement of Hsp70 in BAG3
transcriptional activation, and more broadly, in the triggering
of the stress response, we sought to identify the residue(s)
within the BAG domain that are important for binding to
Hsp70 and assess their ability to augment transcription of the
BAG3 promoter. First, we performed a cross-species conserva-
tion analysis of the primary sequence of the BAG domains of
different members of the family to identify candidate amino
acid residues suitable for mutation and functional analyses. In
this respect, we focused our attention on the highly conserved
third α-helix motif of the BAG domain (Fig. 2A), which is
known for its importance for the interaction of BAG3 with
Hsp70. Among the four highly conserved residues within the
helix α-3 of the BAG domain (shown by asterisks), alterations of
arginine at position 480 into alanine, hereafter called R480A,
impaired the capacity of BAG3 to interact with Hsp70 as tested
by GST pull-down assay (Fig. 2B). Of note, R480A retained its
ability to associate with PLC-γ, which is known to associate with the central proline-rich domain of BAG3 (26).

In an alternative approach to examine the importance of arginine 480 for BAG3 association with Hsp70, mutant cDNA was cloned in pcDNA6-Myc-His, and after its expression in T98G cells, its association with endogenous Hsp70 was determined by immunoprecipitation followed by Western blot using various antibodies that recognize either Hsp70 or tagged protein. As seen in Fig. 2C, in vivo association of R480A with Hsp70 was completely abrogated. As a first step toward understanding a potential engagement of Hsp70 in BAG3 transcriptional activation, T98G glioblastoma cells were transfected with empty vector, wild-type, or R480A BAG3-encoding plasmids and, after 24 h, the nuclear accumulation of HSF1 was measured by Western blot analysis of nuclear protein extracts. HSF1 expression, which correlates with a nuclear accumulation upon wild-type BAG3 overexpression (Fig. 2D, lane 2), was not altered by R480A expression (Fig. 2D, lane 3). Accordingly, results from promoter-reporter assay showed that R480A failed to stimulate the transcription of the BAG3 promoter (Fig. 2E). All these observations point to the importance of helix-3 of BAG3 and residue 480 in the association of BAG3 with Hsp70, nuclear translocation of HSF-1 and auto-regulation of the BAG3 promoter.

HSF1 has been shown to be activated in response to high intracellular levels of ubiquitinated proteins. Proteasome inhibitors such as MG132, ALLN, and lactacystine, trigger the stress...
protein response in several cellular contexts through the activation of Hsp(s) in a HSF1-dependent manner (27, 28).

To understand the molecular events associated with HSF1-driven auto-regulation of BAG3 expression, we sought to determine whether an increase in the level of BAG3 via ectopic expression elevated the overall ubiquitination of BAG3-client proteins. To this end, we first assessed the level of ubiquitinated proteins, which are associated with BAG3 by immunoprecipitation using an antibody that pulls down overexpressed, tagged BAG3 followed by Western blot using anti-ubiquitin antibody. As shown in Fig. 3, a remarkable amount of ubiquitinated immunocomplexes was pulled down from full-length BAG3 but not from ΔC-(1–420)-overexpressing lysates. Deletion of amino acid residues 1–62, which comprise the WW domain, also showed a significant decrease in the amount of ubiquitinated proteins that are immunoprecipitated by ΔC-(1–420)-Myc TAG antibody (Fig. 3C).

FIGURE 2. Binding of BAG3 to Hsp70 is required for BAG3 promoter activation. A, cross-species and cross-members sequence comparison of the evolutionarily conserved helix α-3 of the BAG domain. Human (h), rat (r), mouse (m), and zebrafish (z) amino acid sequences of the BAG domain from BAG3 and from human BAG1 and BAG4 were retrieved as described under “Experimental Procedures.” Representation of aligned sequences was obtained using Clustal W sequence alignment tool. Boxes highlight identical and similar conserved amino acid positions and a clustal consensus is marked by stars. The shaded box points at arginine 480 in the helix α-3 of the BAG Domain of human BAG3. B, in vitro protein interaction assay of wild-type and point mutant BAG3 proteins with Hsc/Hsp70, GST-BAG3, and GST-BAG3(R480A) fusion proteins were incubated with T98G lysate as source of Hsc/Hsp70. Proteins bound to GST-BAG3 (wt or R480A) were identified by precipitation with glutathione-Sepharose beads. The samples were separated by SDS-PAGE and analyzed by Western blot using α-Hsc/Hsp70 and α-PLC. GST alone was used as negative control. C, in vivo association of wild-type and point mutant BAG3 with Hsc/Hsp70. T98G cells were transfected with Myc-His-tagged BAG3 wt or BAG3 (R480A). Cell lysates were immunoprecipitated with an anti-Myc TAG monoclonal antibody or with normal mouse serum (NMS). Immunoprecipitates were analyzed for Hsc/Hsp70 after Western blot using an α-Hsc/Hsp70 monoclonal antibody. Binding of α-His antibody was used as control (NT, not transfected). D, nuclear expression level of HSF1 in R480A transfected T98G cells. Cells were transfected as indicated, and 24 hours later nuclear lysates were prepared. Detection of HSF1 and Lamin A/C was performed as in Fig. 1A and band intensities, respectively, were measured. E, BAG3 promoter activity upon R480A ectopic expression. T98G cells were co-transfected with wild-type BAG3 promoter (−831/+306) plus empty vector or wild-type BAG3 or R480A. Luciferase assay was performed as described in Fig. 2B. The results expressed are the mean of three independent experiments.
unlike wild-type BAG3, the mutant R480A was unable to pull-down ubiquitinated proteins indicating that binding to Hsp70 is required for this event. Interestingly, despite a lower stability of R480A mutant protein in respect to the wild type was observed (supplemental Fig. S1), normalized amounts of immunoprecipitated BAG3 and R480A were used in this experiment to compare the ubiquitinated pulled-down proteins. Moreover, when endogenous BAG3 was pulled down by a rabbit polyclonal antibody, a smear of ubiquitinated proteins was still detected (Fig. 3E).

Because the observed ubiquitination pattern could arise from BAG3 and/or BAG3 clients, we aimed to verify both hypotheses in the next two steps. The direct ubiquitination of BAG3 was evaluated by an in vivo ubiquitination assay. T98G cells were transfected with plasmids encoding HA-tagged wild-type BAG3-(1–576) or ΔC-(1–420) plasmids. Protein lysates were immunoprecipitated with a α-Myc TAG or IgG control antibodies. Immunocomplexes were resolved by SDS-PAGE and immunoblotted with an α-ubiquitin monoclonal antibody (right upper panel) and with α-His TAG polyclonal antibody. Input proteins are shown in the left panel (B). Alternatively cells were transfected with Myc-His-tagged ΔN-(62–576) plasmid. Immunoprecipitation and Western blot analysis were carried out as described in A and B. The asterisks point to the IgG heavy chains.

FIGURE 3. Different domains of BAG3 are involved in ubiquitination of BAG3 partners. A, diagram of BAG3 protein domains. Numbers refer to the corresponding amino acid position. BAG3 overexpression stimulates the accumulation of polyubiquitinated proteins. T98G Cells were transfected with Myc-His-tagged wild-type BAG3-(1–576) or ΔC-(1–420) plasmids. Protein lysates were immunoprecipitated with a α-Myc TAG or IgG control antibodies. Immunocomplexes were resolved by SDS-PAGE and immunoblotted with an α-ubiquitin monoclonal antibody (right upper panel) and with α-His TAG polyclonal antibody. Input proteins are shown in the left panel (B). Alternatively cells were transfected with Myc-His-tagged ΔN-(62–576) plasmid. Immunoprecipitation and Western blot analysis were carried out as described in A and B. The asterisks point to the IgG heavy chains. E, T98G cell lysate was immunoprecipitated with a polyclonal antibody against BAG3 or normal rabbit serum IgG. Western blot was carried out, and the filter was hybridized with a α-ubiquitin (upper panel) or α-BAG3 antibody (lower panel).
pressing Myc-His-BAG3 were treated with 1% SDS as described under “Experimental Procedures” to dissociate BAG3 from its associated proteins, and further subjected to immunoprecipitation with a Myc TAG antibody. Immuno-complexes were analyzed by Western blot using anti-ubiquitin antibody. As shown in Fig. 4A, the smeared ubiquitination pattern was abolished in the presence of denaturing agents (compare lanes 2–4), indicating that BAG3 partners are ubiquitinated in this event and that their contribution to the ubiquitination pattern is remarkable. Of note, equal amounts of BAG3 immunocomplex were used in these experiments (Fig. 4B, middle panel, compare lanes 2 and 4) and protein-protein interaction was further verified (Fig. 4B, lower panel).

The ubiquitin lysine-less (KØ) mutant isoform is impaired in the ability to form polyubiquitin chains on target proteins of the UPS (ubiquitin proteasome system) and serves as a powerful tool to evaluate the ubiquitination dependence of the target degradation (27). Co-transfections of BAG3 isoforms with HA-tagged wt- or lysine-less (KØ)-ubiquitin were carried out in T98G cells and total lysates were immunoprecipitated with.

**FIGURE 4.** Ubiquitination occurs both on BAG3 and BAG3-client proteins. A, analysis of BAG3 ubiquitination in vivo. T98G cells were transfected with HA-tagged ubiquitin in the presence of an empty vector (lanes 1 and 3) or BAG3 tagged at the C terminus with Myc-His (lanes 2 and 4). Cell extracts were immunoprecipitated with a monoclonal antibody against HA (lanes 3 and 4). Immunoprecipitates were analyzed by immunoblotting with an α-His antibody. Arrows point to ubiquitinated isoforms of BAG3, whereas the asterisks point to the canonical isoform. B, evaluation of BAG3 substrates ubiquitination in vivo. T98G cells expressing Myc-His-tagged BAG3 were harvested and lysed in native conditions (lanes 1 and 2) or in the presence of 1% SDS denaturing conditions (lanes 3 and 4) and subsequently immunoprecipitated with a monoclonal antibody against Myc TAG or control IgG. Immunocomplexes were immunoblotted with an α-ubiquitin antibody (upper panel). α-His and α-Hsc/Hsp70 hybridization served as immunoprecipitation and partner-binding controls, respectively.

C, inhibition of polyubiquitin chain formation stabilizes ubiquitinated BAG3 partners and BAG3 itself. T98G cells expressing Myc-His tagged BAG3 or ΔC(1–420) or R480A mutant plus HA-tagged ubiquitin or lysine-less ubiquitin (KØ), were immunoprecipitated with α-Myc TAG antibody (upper panel) or with α-HA antibody (second panel from the top). Immunocomplexes were analyzed by Western blot and hybridized with α-ubiquitin and α-His TAG, respectively. His TAG and α-tubulin immunoblots from 20 μg of input lysates are shown in lower panels.

D, proteasomal inhibition stabilizes ubiquitinated BAG3 partners. In vivo immunoprecipitation assay using α-Myc TAG antibody or control IgG with T98G lysate co-expressing Myc-His-tagged BAG3 and HA-tagged ubiquitin (lanes 1 and 2), plus 5 h of treatment with pepstatin A (lanes 3 and 4), or co-expressing Myc tagged BAG3 and ubiquitin (KØ) (lanes 7 and 8). Immunoblot of immunoprecipitated complexes was reacted with an ubiquitin antibody (upper panel). The input levels of ectopic BAG3 are shown in the lower panel. The asterisks point to the IgG heavy chains (B, C, and D).
α-Myc TAG or α-HA antibodies. The co-expression of Ub(KO) determined a remarkable increase in the amount of ubiquitinated clients bound to wt BAG3, but not to ΔC-(1–420) or R480A (Fig. 4C, upper panel). Likewise, wt BAG3 pulled down by α-HA antibody was markedly augmented in presence of Ub(KO) (Fig. 4C, lower panel). The basal ubiquitination levels of both clients and BAG3, in the presence of wt-ubiquitin, were even more evident with a longer exposure of the film (Fig. 4C, right panels).

Interestingly, additional accumulation of ubiquitinated BAG3-clients, following BAG3 overexpression, was observed when T98G were treated for 5 h with the proteasomal inhibitor MG132 but not with the lysosomal protease inhibitor Pepstatin A (Fig. 4D), indicating that proteasomal activity is important for the clearance of the ubiquitinated proteins.

The accumulation of ubiquitinated proteins is an indication of a stressful condition for the cell. Different insults can induce extensive damage at the protein level. This could result in the triggering of a stress protein response that could potentially lead to unfavorable growth conditions and eventually to the cell death.

To investigate the impact of BAG3 overexpression on cell growth, we set up a series of anchorage-dependent and -independent colony formation assays. T98G cells were transfected with an equimolar amount of plasmids encoding different BAG3 isoforms and carrying the selection marker for blasticidin, which confers resistance to the corresponding eukaryotic antibiotic. After replating, cells were cultured in medium supplemented with blasticidin for 15–20 days, with periodic medium change every 3 days. Colonies originating from single cells were stained and counted. As shown in Fig. 5A, wild-type BAG3 expression resulted in a remarkable drop in colony number when compared with pcDNA6 empty vector-transfected cells. Notably, deletion of C-terminal part of the protein (ΔC-(1–420) displayed a comparable number of colonies as the empty vector. Quantitatively the expression of wt BAG3 resulted in a 88% and a 79% decrease in colony number compared with empty vector control and ΔC-(1–420), respectively. Similar results were obtained in anchorage-independent conditions (~78% in BAG3 wt-overexpressing cells) as shown by colonies growing in soft agar (Fig. 5B). Interestingly, the R480A mutant expres-
sion partly restored the number of colonies observed in the control, indicating that Hsp70 binding activity correlates with the tumor suppressor-like phenotype described. Comparable results were obtained in other glioblastoma cell lines such as U-87MG and LN229 but not in non glial-derived cell lines such as HeLa and HEK293 (data not shown).

**DISCUSSION**

Heat shock and other proteotoxic stresses activate BAG3 in an HSF1-dependent manner (11, 12, 14, 29). From this point of view, the autoregulatory feedback involving BAG3 promoter activation appears to be the consequence of a stress response generated by BAG3 overexpression, as the mutation of HSE in the 5'-UTR DNA region prevents the up-regulation of BAG3 transcription (Fig. 1B).

Nuclear translocation of HSF1 (Fig. 1A) in glioblastoma cells overexpressing BAG3 mimics a heat shock/protein stress response, as also indicated by the Hsp70 transcript accumulation (Fig. 1C). Accordingly, endogenous BAG3 levels sustain HSF1-target genes expression such as Hsp70 and Hsp27 (Fig. 1D). This last result is in line with our previous observation demonstrating that down-modulation of the BAG3 endogenous transcript resulted in a decreased transcription rate of BAG3 promoter in glioblastoma cell lines (22).

Our results indicate that binding of BAG3 to Hsp70 plays a critical role in triggering the stress response. Indeed, HSF1 nuclear translocation and, eventually, BAG3 promoter activation remain unchanged upon R480A overexpression. Accordingly, identification of another Hsp70-binding mutant (Q488A), which has a lower affinity for Hsp70 than wild-type BAG3, showed a decreased stimulatory effect on BAG3 promoter (data not shown). Nevertheless, the conformational change determined by R480A substitution remains unclear. Interestingly, the mutation of the corresponding arginine in the multiple BAG domains of BAG5, in combination with the alteration of another residue (also conserved in BAG3), impairs both the binding to Hsp70 and BAG5 self-association (30).

The activation of HSF1 by BAG3 overexpression correlates to a dramatic increase of ubiquitinated proteins bound to BAG3 and this was also verified to a lesser extent for endogenous BAG3. These observations corroborate earlier results demonstrating that elevated levels of ubiquitinated proteins enhance BAG3 gene transcription through a regulatory loop involving HSF1 and the HSE motif within the BAG3 5'-UTR (14). HSF1 is known to be sensitive to the folded state of intracellular proteins, and in the presence of a stressful condition, which affects protein conformation and functionality, HSF1 is activated and it eventually triggers the stress response transcriptional program (3). Ubiquitination is the main signal utilized by the cell to tag the proteins that must be cleared out and this molecular event described to be influenced by the WW domain of BAG3.

In a recent study, Gamerdinger et al. (17) investigated the role of BAG3 in the stimulation of the autophagic pathway during brain aging as they correlated this process to an adaptation of the PQC to maintain the protein homeostasis (17). While the lysosomal degradation of oligomeric and aggregated proteins that are impossible to unfold is crucial for protein clearance, proteasomal degradation of a specific target requires a tightly regulated series of events that span from the target recognition to the connection to the UPS.
Evidence in support of BAG3 involvement in the lysosomal-dependent disposal of aggregated proteins was also provided by Carra et al. (18) who verified that the clearance activity of BAG3 toward polyQ-containing protein aggregates is associated with the stimulation of the macropathic pathway. Our results show, for the first time, a connection with BAG3 and the proteasome. In light of these findings, it is tempting to hypothesize that BAG3 can recognize and, in combination with other cellular machineries, target specific clients to the proteasomal degradation pathway to facilitate their turnover.

From this perspective, BAG3 could be imagined as an ubiquitination platform that connects the Hsp70-dependent ubiquitination machinery and protein substrates selectively recognized by the interaction with other regions of BAG3 such as the WW domain. Another possible explanation to this event could reside in the ability of BAG3 to inhibit the Hsp70 folding activity. As shown by in vitro studies, several BAG proteins, including BAG3, retain the ability to negatively regulate Hsp70 in refolding a denatured luciferase protein. Interestingly this inhibition is dependent on the presence of the BAG domain (13). In light of this consideration the inhibition of Hsp70-folding activity may result in an accumulation of unfolded proteins that need to be targeted for proteasomal degradation.

Surprisingly, ectopic expression of wild-type BAG3 has a negative impact on cell growth and this effect is mediated by an Hsp70-dependent mechanism (Fig. 5, A and B). This new property of BAG3 was only observed in glioblastoma cell lines and not in non-gli-al-derived cells such as HeLa and MCF7. Most recently, Jung et al. (32) demonstrated that BAG3 knockdown in rat glioma cells sensitizes cell death in response to stress. These observations corroborate our recent preliminary results from colony formation assays showing that downmodulation of BAG3 affects cell growth and viability (data not shown). Altogether, these observations suggest that in addition to its role in promoting cell survival, BAG3 at different levels may have a positive or negative impact on the growth of glioblastoma cells. Of note, overexpression of BAG3 in HeLa cells did not result in HSF1 nuclear translocation and massive accumulation of polyubiquitinated proteins (Supplemental Fig. S4) such as glioblastoma cell lines. The specificity of clients could justify the difference in the phenotypic outcome of BAG3 expression in glioblastoma cells and non-gli-al cell lines. Indeed, our earlier study has shown that BAG3 displays a nuclear-cytoplasmic localization in glioblastoma cells compared with the most commonly described cytoplasmic distribution in HeLa and other cell lines (2, 11). A putative nuclear client could engage the BAG3 partnership in glioblastoma cells and could be processed by the machinery recruited by BAG3. Another possible explanation could arise from the striking increase in ubiquitinated proteins following BAG3 expression that in turn stimulates HSF1 activity and eventually the activation of stress response genes that could finally slow down cell growth.

There is an accumulating body of evidence that clearance of protein aggregates, deriving from aged or damaged proteins, is impaired in neurodegenerative disorders such as Parkinson and Alzheimer diseases (33). The identification of BAG3 as a new player in the Protein Quality Control pathway and in the turnover of proteins will give new insights into the etiology of neurodegenerative diseases and will provide new therapeutic targets.

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