BAG-1 Prevents Stress-induced Long-term Growth Inhibition in Breast Cancer Cells via a Chaperone-dependent Pathway

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ABSTRACT

BAG-1 is a multifunctional protein that interacts with a wide range of cellular targets. There is accumulating evidence that overexpression of BAG-1 may play an important role in breast cancer; however, the functional consequences of BAG-1 expression and its mechanism of action in breast cancer cells have not been studied in detail. Here we demonstrate that BAG-1 overexpression completely protected breast cancer cells from apoptosis and long-term growth inhibition induced by heat shock and also partially protected cells from other stresses, including hypoxia, radiation, and chemotoxic drugs. BAG-1 exists as three protein isoforms, and all isoforms prevented stress-induced growth inhibition. This required a conserved lysine in the BAG-1 ubiquitin-like domain thought to be important for proteasome binding and COOH-terminal amino acids required for interaction with the chaperone molecules, Hsc70 and Hsp70. Although expression of BAG-1 was unaffected by heat shock, endogenous and overexpressed BAG-1 was relocalized from the cytoplasm to the nucleus after heat shock. The endogenous BAG-1/Hsc70/Hsp70 complex dissociated after heat shock but was maintained at a detectable level in cells overexpressing BAG-1. BAG-1-mediated resistance to stress-induced growth inhibition is likely to have a major impact on the development and response to therapy of breast cancer. Targeting the interaction of BAG-1 with chaperones is an attractive strategy to counter the biological effects of BAG-1.

INTRODUCTION

BAG-1 is a multifunctional protein that interacts with a wide range of cell targets and regulates cell survival, signaling, metastasis, proliferation, and transcription (1–5). Cells express several BAG-1 isoforms through alternate translation initiation of a single mRNA (6–9). The most abundant isoform, p36 BAG-1S, is translated from an AUG codon and is a predominantly cytoplasmic protein. The largest isoform, p50 BAG-1L, is translated from an upstream CUG codon and, consistent with the presence of a NLS4 within its NH₂-terminal extension, resides within the cell nucleus. The other isoform, p46 BAG-1M, is generally expressed at low levels in human cells and is not detected in other species. Various domains have been recognized within BAG-1 isoforms, including a ULD and a COOH-terminal evolutionarily conserved BAG domain (10, 11). The structure of the human BAG-1 isoforms is shown in Fig. 5.

BAG-1 protects cells from a wide range of apoptotic stimuli, including Fas, cytotoxic drugs, staurosporine, heat shock, and growth factor withdrawal (1, 10, 12–15). However, the mechanisms responsible for these effects remain unclear. BAG-1 proteins interact and modulate the activity of the M, 70,000 heat shock proteins, Hsc70 and Hsp70, via the COOH-terminal BAG domain (16–20). The prosurvival effects of BAG-1 have often been ascribed to this interaction because Hsp70 and Hsc70 play important roles in regulating cell survival (21, 22), and deletion of the BAG-1 COOH terminus abrogates its prosurvival activity, as well as many other BAG-1 functions (12, 23–25). It is important to recognize, however, that the BAG-1 COOH terminus is also required for interaction and activation of Raf-1 (26), a serine-threonine kinase involved in cell survival and proliferation that provides an alternate potential mechanism by which BAG-1 might promote cell survival (27). Hsp70 and Raf-1 compete for binding to BAG-1, and high levels of Hsp70 prevent activation of Raf-1 by BAG-1 in hamster fibroblasts, suggesting that growth-and/or survival-promoting effects of BAG-1 may be mediated by activation of Raf-1-dependent mitogen-activated protein kinase pathways and negatively regulated by chaperone binding (28).

BAG-1 also interacts with the proteasome and has been suggested to act as bridge to link chaperone molecules with the proteasome, the major nonlysosomal protease in cells (29, 30). Although precise protein regions required for interaction are not known, binding does require the BAG-1 NH₂ terminus that contains a ULD, similar to ubiquitin and the ULD found in other proteins such as Rad23 (10, 31). The Rad23 ULD mediates binding to the proteasome, strongly suggesting that BAG-1 binding to the proteasome is also mediated via its ULD. Although BAG-1 NH₂-terminal deletions inactivate the survival functions of BAG-1 (12, 23), the role of specific amino acid residues within the ULD has not been determined.

BAG-1 expression is frequently altered in human cancer (2, 32–36). In breast cancer, overexpression of nuclear or cytoplasmic BAG-1 protein has been detected in the majority of cases, and this can correlate with clinicopathological features and clinical outcome (2, 32–34). Elevated levels of cytoplasmic BAG-1 expression are associated with improved prognosis in early-stage breast cancer independent of nodal status, suggesting that BAG-1 may have clinical utility as a prognostic marker (33). We have also shown that nuclear BAG-1 expression is associated with improved survival in patients treated with hormonal therapy. By contrast, Tang et al. (34) demonstrated that increased expression of BAG-1 was associated with a poorer outcome. These findings suggest that BAG-1 overexpression plays an important role in the development and response to therapy in breast cancer.

Despite these encouraging findings, the functional consequences and mechanism of action of BAG-1 in breast cancer cells have not been studied in detail. Here we demonstrate that BAG-1 overexpression has profound effects on the long-term survival of breast cancer cells exposed to cellular stresses, mediated via interaction with chaperone molecules.

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4 The abbreviations used are: NLS, nuclear localization sequence; ULD, ubiquitin-like domain; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FCNA, proliferating cell nuclear antigen; HA, hemagglutinin; PARP, poly(ADP-ribose) polymerase.
BAG-1 FUNCTION IN BREAST CANCER CELLS

MATERIALS AND METHODS

Cell Culture and Transfections. MCF7 human breast cancer cells were maintained in DMEM (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% (v/v) FCS (PAA Laboratories, Yeovil, United Kingdom) and penicillin and streptomycin (Life Technologies, Inc.). To produce stable clones overexpressing human BAG-1S, MCF7 cells were transfected with pcDNA3-BAG-1S expression construct or empty pcDNA3 vector (Invitrogen) using calcium phosphate precipitation followed by single cell cloning in the presence of G418. All other transfections were performed using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions.

Cells were exposed to stress by heat shocking [42°C for 1 h in a Hybaid Micro-4 microhybridization oven (Hybaid)], hypoxia [24 h, 0.3% O2 using an anaerobic chamber (Oxoid, Basingstoke, United Kingdom)], or ionizing radiation. G6PDH and BAG-1 were exposed to heat shock, radiation, or chemotherapeutic drugs [cisplatin (10 μg/ml), paclitaxel (1.6 μM), doxorubicin (50 μM), etoposide (30 μM), or vincristine (1.6 μM)] for 24 h. These “levels” of stress (i.e., temperature, time of hypoxia, amount of radiation, and concentration of cytotoxic drug) were selected to give approximately equivalent levels of growth reduction (70–90%) in long-term clonogenic assays. Cells were allowed to recover at 37°C after heat shock, or after washing to remove drugs, before analysis.

Long-term Growth Assays. Long-term growth assays were performed in two ways, using MCF7 cell-derived clones stably overexpressing BAG-1S or after transient transfection of BAG-1 isoform or mutant expression constructs. In the first assay, stably transfected BAG-1S-overexpressing or pcDNA3 control clones were exposed to heat shock, allowed to recover for at least 16 h, and then serially diluted 10-fold in 24-well plates. Cells were cultured for 21–28 days to allow colonies to form. In the second assay, parental MCF7 cells were transiently transfected with control or BAG-1 expression constructs and allowed to recover overnight. Transfected cells were then exposed to heat shock, hypoxia, radiation, or chemotherapeutic drugs; allowed to recover for at least 16 h; and then serially diluted 10-fold in 24-well plates. Cells were cultured for 21–28 days (in the presence of G418 to eliminate untransfected cells) to allow colonies to form. Plates were rinsed with PBS and fixed for 7 min with methanol. The plates were allowed to air dry for 10 min followed by the addition of Wright-Giemsia stain (Sigma) for 3 min. The plates were washed in distilled water and then allowed to air dry. Colonies were counted using a colony counter.

TRAIL-induced Apoptosis. Cells were incubated with recombinant FLAG-tagged TRAIL (100 or 250 ng/ml) and an anti-FLAG antibody (both from Alexis Biochemicals) to enhance receptor cross-linking for 3 days. Cell viability was measured using the Cell Titre 96 Aqueous CellProliferation assay (Promega).

Fluorescence-activated Cell Sorting. Cells were collected by trypsinization, washed in PBS, and fixed in 70% (v/v) ethanol. Fixed cells were collected by centrifugation and incubated with propidium iodide (0.05 mg/ml) and RNase A (0.1 mg/ml) in PBS for 60 min at room temperature in the dark. DNA content was determined by flow cytometry using a FacsCalibur (Becton Dickinson). The proportion of cells with sub-G1 content as a percentage of total cells was determined.

Plasmids. Deletion and site-directed mutagenesis were used to generate pcDNA3-derived plasmids that uniquely expressed human BAG-1S, BAG-1M, or BAG-1L.3 BAG-1S deletion mutants were generated by PCR using the following primers: (a) BAG-1S574-230; 5’-GCCACGATGGATTGAAATCACA and 5’-TCTACACCTACTCGGCACGGG; (b) BAG-1S1-165, 5’-ACCCGGGGCGAGATGATCTCGG and 5’-TCAGCTCTAGCTGC- AAATC; and (c) BAG-1S1-112, 5’-ACCCGGGGCGAGATGATCTCGG and 5’-TGTACTACCTACTCGGCACGGG. PCR products were TA-cloned into the pCR-TOPO vector (Invitrogen), verified by sequencing, and subcloned into pcDNA3 (Invitrogen). Wild-type mouse BAG-1S and mouse BAG-1S120K mutant were a kind gift of Prof. Richard I. Morimoto (Northwestern University, Evanston, Chicago, IL) and express HA-epitope-tagged proteins.

Common precipitation and Immunoblotting. Cells were resuspended in 1 ml of HMK buffer Ref. 14; 10 mM n-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.2), 5 mM MgCl2, 1/2 mM KCl, 2 mM ethylene glycol-bis-(β-aminopropyl ether)-N,N’,N”-tetraacetic acid, 0.2% (v/v) NP40, and Protease Inhibitor Cocktail (Sigma) by trituration through a 21-gauge needle, lysed on ice for 30 min, and clarified by centrifugation (12,000 rpm for 10 min in a microfuge). Ten percent of the lysate was retained as a whole cell (input) lysate. The remaining sample was precleared using protein G-Sepharose beads (Amersham Pharmacia Biotech) for 30 min at 4°C. Protein G-Sepharose beads were removed by centrifugation. For human BAG-1 immunoprecipitations, the lysate was divided and incubated with the BAG-1-specific rabbit polyclonal antibody TB2 (7) or with preimmune control serum (both at 5 μl/450 μl lystate). Mouse BAG-1 was immunoprecipitated using the HA-epitope tag-specific mouse monoclonal antibody F7 (Santa Cruz Biotechnology) or purified mouse IgG (BD Pharmingen) as a control (both at 1.8 μg/ml). After 5 (mouse BAG-1) or 16 (human BAG-1) at 4°C, the lystate was incubated with protein G-Sepharose beads for 1 h, and immunocomplexes were removed by centrifugation. The beads were washed five times using HMK buffer, resuspended in SDS-PAGE sample buffer, and heated at 95°C for 5 min. Subcellular fractions were prepared as described previously (7). All nuclear fractions contained <5% cytoplasmic contamination as measured using lactate dehydrogenase activity (data not shown).

Immunoblotting was performed as described previously (6) using mouse monoclonal antibodies 3.10 G3E2 and 3.10 G3F1 or rabbit polyclonal antibodies TB2 (7) and C-16 (Santa Cruz Biotechnology) for human BAG-1, rabbit polyclonal antibody m10 (6) for mouse BAG-1, the Hsc70-specific mouse monoclonal antibody B6 (Santa Cruz Biotechnology), Hsp70-specific mouse monoclonal antibody (Stressgen), or the PARP-specific mouse monoclonal antibody C-20 (R&D Systems).

Immunofluorescence. Cells were cultured overnight on FCS-coated glass coverslips, washed twice in PBS, and fixed in PBS containing 4% (w/v) paraformaldehyde for 15 min at room temperature. Cells were washed with PBS and incubated with PBS containing 10% (v/v) newborn calf serum and 0.05% (w/v) sodium azide for 20 min at 4°C. Cells were washed and permeabilized by incubation in permeabilization buffer [PBS containing 10% (v/v) newborn calf serum and 1% (v/v) Triton X-100] for 20 min at room temperature. Cells were washed and then incubated with the polyclonal anti-BAG-1 antibody TB2 (7) diluted 1:1000 in permeabilization buffer for 20 min at room temperature. Cells were washed and incubated with 2 μg/ml FITC-conjugated antirabbit immunoglobulins (Sigma) diluted in permeabilization buffer for 1 h at room temperature. Coverslips were then washed in PBS and mounted on glass slides using Fluorescent Mounting Media (Dako). Cells were visualized on an Axiovert 100 fluorescence microscope (Zeiss).

RESULTS

Effect of BAG-1 on Stress-induced Apoptosis and Long-term Growth Inhibition. To analyze the effects of BAG-1 overexpression on cell survival in breast cancer cells, we generated MCF7 cell-derived clones stably overexpressing human BAG-1S (Fig. 1A). We used MCF7 cells because they have an intermediate level of endogenous BAG-1 expression, and initial experiments focused on heat shock as a well-studied model system for cellular stress. We first analyzed apoptosis in short-term assays by determining the proportion of cells with fragmented DNA and cleavage of PARP. Although MCF7 cells lack caspase 3, PARP is degraded by other proteases during apoptosis (37). In control cells, there was a decrease in the level of full-length PARP and an increase in the proportion of cells with sub-G1 DNA content after heat shock, consistent with induction of apoptosis (Fig. 1, B and C). BAG-1S overexpression prevented induction of apoptosis after heat shock.

It is not known whether BAG-1 prevents or simply delays the onset of apoptosis, and it was important therefore to determine whether BAG-1 also protected cells from heat shock in long-term assays. Whereas heat shock reduced the long-term growth potential of control cells by approximately 90%, BAG-1S-overexpressing clones were almost completely protected from growth inhibition (Fig. 1D). There-
fore, BAG-1S overexpression enables long-term growth after heat shock in breast cancer cells.

We tested whether BAG-1 inhibited long-term growth inhibition induced by other cellular stresses. In this assay, parental MCF7 cells were transfected with BAG-1S expression construct or empty vector as a control. Sixteen h after transfection, cells were exposed to γ-radiation, hypoxia, or chemotherapeutic drugs and allowed to recover overnight. Cells were then serially diluted, and the long-term growth potential of successfully transfected (i.e., G418-resistant) cells was determined. BAG-1S overexpression partially suppressed long-term growth inhibition induced by γ-radiation, hypoxia, and paclitaxel, doxorubicin, etoposide, and vincristine, although to a lesser extent than heat shock (Fig. 2). BAG-1S did not affect growth inhibition induced by cisplatin. Therefore, the protective effects of BAG-1 are not restricted to heat shock, and BAG-1 protects cells from the growth-inhibitory effects of a wide range of cellular stresses.

Effect of BAG-1 on TRAIL-induced Apoptosis. We analyzed whether BAG-1S overexpression prevented apoptosis induced by ligation of cell surface TRAIL receptors to determine whether the profound effects of BAG-1 on stress-induced cell killing reflected a more general effect on apoptosis per se. Control pcDNA3-transfected clones were sensitive to TRAIL-induced apoptosis, with an average reduction in cell growth of 72 ± 2% after treatment with 100 ng/ml TRAIL (Fig. 3). BAG-1S overexpression reduced TRAIL-induced apoptosis with the lowest concentration of TRAIL tested (mean = 53 ± 4% growth inhibition). However, BAG-1S-overexpressing cells were still sensitive to TRAIL-induced apoptosis, and the protective effects of BAG-1 were overcome by increased amounts of TRAIL (80% versus 81% growth inhibition for control and BAG-1S-overexpressing cells at 250 ng/ml TRAIL).

Analysis of BAG-1 Isoforms and Mutants. Cells express three major BAG-1 isoforms, and we compared their ability to overcome long-term growth inhibition induced by heat shock. We used the transient transfection-based assay described above because it allowed us to perform titration experiments to compare the effects of different levels of expression of BAG-1 as well as more readily determine the effects of combinations of wild-type and mutant BAG-1 proteins. Moreover, this approach circumvented problems associated with generating stable clones expressing potentially growth-inhibitory BAG-1 mutants (25).

We generated human BAG-1 isoform-specific expression constructs and demonstrated that the constructs expressed the expected protein products at approximately equivalent levels. Coimmunoprecipitation experiments confirmed that all BAG-1 isoforms interacted to an equivalent level with Hsc70 and Hsp70 (Fig. 4A). Similar to the stably transfected MCF7 clones (Fig. 1D), heat shock reduced the clonogenic potential of MCF7 cells transfected with pcDNA3 by approximately 90%, and this was significantly inhibited by expression of high levels of BAG-1S (Fig. 4B). Titration experiments using different amounts of expression constructs demonstrated that the extent of rescue was dose dependent, and the three BAG-1 isoforms were approximately equally effective in rescuing cells from growth inhibition. Consistent with the dose response observed in long-term growth assays in MCF7 cells, we demonstrated that expression of BAG-1 from the isoform expression plasmids was also titratable and equivalent between isoforms over this range of plasmid
Parental MCF7 cells were transfected with 100 ng of wild-type and/or 100 ng of mutant BAG-1S expression plasmids as indicated (per well of a 24-well plate). Empty pcDNA3 plasmid was used to ensure that each transfection contained a constant total DNA content (2 μg of wild-type and/or 1 μg of mutant BAG-1S expression plasmids as indicated (per 90-mm plate). Empty pcDNA3 plasmid was used to ensure that each transfection contained a constant total DNA content (2 μg). BAG-1 was detected by immunoblotting using BAG-1-specific polyclonal antibody TB2 or preimmune serum (PI) as a control where indicated. Protein samples were analyzed for BAG-1 expression using BAG-1-specific monoclonal antibodies that recognize distinct epitopes. A mixture of monoclonal antibodies that recognize distinct epitopes was used to detect the BAG-1 truncation mutants. D, long-term growth assay in MCF7 cells transfected with the indicated wild-type and mutant BAG-1S expression plasmids. Parental MCF7 cells were transfected with 100 ng of wild-type and/or 100 μg of mutant BAG-1S expression plasmids as indicated (per well of a 24-well plate). Empty pcDNA3 plasmid was used to ensure that each transfection contained a constant total DNA content. Sixteen h after transfection, cells were exposed to transient heat shock or left untreated as a control. Cells were allowed to recover and serially diluted, and colonies were allowed to form in the presence of G418. The graph shows the number of colonies (mean ± SE) from three independent experiments performed in triplicate. Parental MCF7 cells were transfected with pcDNA3 or BAG-1 isoform-specific expression constructs (2 μg of wild-type and/or 1 μg of mutant BAG-1S expression plasmids (0.1, 1, and 10 ng/well of a 24-well plate)). Sixteen h after the transfection, cells were exposed to transient heat shock or left untreated as a control. Cells were allowed to recover and serially diluted, and colonies were allowed to form in the presence of G418. The graph shows the number of colonies (mean ± SE) from three independent experiments performed in triplicate. Parental MCF7 cells were transfected with 100 ng of wild-type and/or 100 ng of mutant BAG-1S expression plasmids as indicated (per well of a 24-well plate). Empty pcDNA3 plasmid was used to ensure that each transfection contained a constant total DNA content (2 μg). BAG-1 was detected by immunoblotting using BAG-1-specific polyclonal antibody TB2 or preimmune serum (PI) as a control where indicated. Protein samples were analyzed for BAG-1 expression using BAG-1-specific monoclonal antibodies that recognize distinct epitopes. A mixture of monoclonal antibodies that recognize distinct epitopes was used to detect the BAG-1 truncation mutants. D, long-term growth assay in MCF7 cells transfected with the indicated wild-type and mutant BAG-1S expression plasmids. Parental MCF7 cells were transfected with 100 ng of wild-type and/or 100 μg of mutant BAG-1S expression plasmids as indicated (per well of a 24-well plate). Empty pcDNA3 plasmid was used to ensure that each transfection contained a constant total DNA content. Sixteen h after transfection, cells were exposed to transient heat shock or left untreated as a control. Cells were allowed to recover and serially diluted, and colonies were allowed to form in the presence of G418. The graph shows the number of colonies (mean ± SE) from three independent experiments performed in triplicate. Parental MCF7 cells were transfected with 100 ng of wild-type and/or 100 ng of mutant BAG-1S expression plasmids as indicated (per well of a 24-well plate). Empty pcDNA3 plasmid was used to ensure that each transfection contained a constant total DNA content. Sixteen h after transfection, cells were exposed to transient heat shock or left untreated as a control. Cells were allowed to recover and serially diluted, and colonies were allowed to form in the presence of G418. The graph shows the number of colonies (mean ± SE) from three independent experiments performed in triplicate. Parental MCF7 cells were transfected with 100 ng of wild-type and/or 100 ng of mutant BAG-1S expression plasmids as indicated (per well of a 24-well plate). Empty pcDNA3 plasmid was used to ensure that each transfection contained a constant total DNA content. Sixteen h after transfection, cells were exposed to transient heat shock or left untreated as a control. Cells were allowed to recover and serially diluted, and colonies were allowed to form in the presence of G418. The graph shows the number of colonies (mean ± SE) from three independent experiments performed in triplicate.

concentrations.6 These experiments were performed in HEK 293 cells, which have very low endogenous BAG-1 expression. It was not possible to perform this analysis in MCF7 cells because the relatively high levels of endogenous BAG-1 proteins masked low-level exogenous expression.

Because protein sequences within BAG-1S were sufficient to counter effects of stress, we created deletion mutants to determine which parts of BAG-1S were required. The BAG-1S NH2-terminal ULD contains a lysine residue (Lys80 in human BAG-1S) that is conserved in ubiquitin and other ubiquitin-like proteins (10), and we also created an alanine substitution mutant of this residue (BAG-1SK80A; Fig. 5A). Immunoblot analysis and coimmunoprecipitations from transiently transfected MCF7 cells were used to analyze expression of the mutants and their association with chaperones. Immunoblotting with antibody C-16, which recognizes a COOH-terminal epitope, detected wild-type BAG-1S, BAG-1S89–230, and BAG-1SK80A, but not BAG-1S1–155, at approximately equivalent levels (Fig. 5B). Antibody TB2, raised against BAG-1S, recognized BAG-1S, but not BAG-1S89–155. When tested in long-term assays, all mutants were defective in preventing heat shock-induced growth inhibition (Fig. 5D).

We tested whether the nonfunctional mutants interfered with the

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activity of the wild-type molecule (Fig. 5D). Coexpression of BAG-1S1–155, BAG-1S89–230, or BAG-1S89–230A partially abrogated the protective effects of BAG-1S, demonstrating that they can act in a transdominant fashion to inhibit BAG-1 function. Coexpression of the mutants did not interfere with expression of wild-type BAG-1S or association with Hsc70/Hsp70 (Fig. 5, C and D). Interestingly, both the BAG-1S89–230 and BAG-1S89–230A mutants containing an intact BAG domain gave a modest decrease in the clonogenic potential of the control (nontreated) MCF7 cells (Fig. 5B), suggesting that endogenous BAG-1 is important for the normal growth of these cells. This effect was reproducible but failed to reach statistical significance.

**Regulation of BAG-1 Localization and Interaction after Heat Shock.** BAG-1 isoforms are differentially localized in breast cancer cells, with BAG-1S located predominantly in the cytoplasm, and BAG-1L located predominantly in the nucleus (7). It was surprising therefore that these isoforms were equally effective at protecting cells from stress. However, the localization of BAG-1 proteins can be regulated (1, 38), and we therefore determined the effect of heat shock on BAG-1 localization in MCF7 cells. Consistent with previous reports, BAG-1S was predominantly present in the cytoplasm of control MCF7 cells (Fig. 6A). Although the expression of BAG-1 isoforms was not altered for up to 16 h after heat shock,6 BAG-1S was largely excluded from the cytoplasm to the nucleus, and after 4 h of recovery at 37°C, approximately half of the BAG-1S was detected in the nuclear fraction. As expected (39), Hsc70 also relocalized to the nucleus. The accumulation of endogenous BAG-1 in the nucleus after heat shock was confirmed by immunofluorescence (Fig. 6B). Similar results were obtained after analysis of BAG-1S-overexpressing MCF7 cell-derived clones, with nuclear accumulation of BAG-1 without alterations in expression levels.6

We also studied the interaction of BAG-1 protein with chaperones after heat shock. Interaction of endogenous BAG-1 with Hsc70 and Hsp70 was readily detectable in control MCF7 cells but was reduced below the level of detection at 4 h after heat shock (Fig. 6C). Overexpression of BAG-1S in the stable clones increased chaperone binding in cells maintained at 37°C, and, although also relatively reduced, interactions between BAG-1 and chaperones were maintained after heat shock. Therefore, heat shock triggers dissociation of BAG-1 and Hsc70/Hsp70 in control cells, but binding is maintained by high levels of BAG-1S.

**Site-directed Mutagenesis of the BAG Domain.** The BAG-1 COOH terminus interacts with Hsc/Hsp70 and Raf-1 through overlapping yet separable binding sites (28). To test the relative contribution of chaperones and Raf-1 binding for BAG-1 function, we used a murine BAG-1S point mutant (E208A; a kind gift of Prof. Richard I. Morimoto) that is specifically defective in binding to chaperones yet retains the ability to bind and activate Raf-1 (28). If BAG-1 promotes growth via Raf-1, one would expect this mutant to be active or possibly hyperactive because it would not be subject to negative regulation by Hsc/Hsp70. In contrast, if Hsc/Hsp70 binding is essential for BAG-1 function, then this mutant would be nonfunctional. Similar to human BAG-1S, mouse BAG-1S effectively suppressed long-term growth inhibition (Fig. 7A). By contrast, the BAG-1S COOH-terminal point mutant was completely inactive, although it was expressed at equivalent levels (Fig. 7B). The failure of this protein to interact with Hsc70 was confirmed in coimmunoprecipitation experiments in MCF7 cells (Fig. 7B). Therefore, binding to Hsc/Hsp70 is required to suppress growth inhibition. A second mouse BAG-1S mutant (C204A) deficient in chaperone binding (28) was also unable to suppress long-term growth inhibition; however, the expression of this protein in MCF7 cells was significantly reduced compared with wild-type murine BAG-1S.6

**DISCUSSION**

There is compelling evidence that BAG-1 expression is altered in breast cancer, and this may impact on patient survival (2, 32–34). However, the functional consequences of BAG-1 overexpression and mechanism of action in breast cancer cells have not been studied in detail. Previously, BAG-1 has been demonstrated to interfere with the growth-inhibitory effects of retinoic acid and to delay apoptosis after serum withdrawal and stimulate tumor growth of ZR-75-1 cells (25, 40). We have now shown that BAG-1 overexpression also has profound effects on the response to stress in breast cancer cells, preventing apoptosis induced by heat shock. This is the first demonstration that BAG-1 protects cells in long-term assays. Importantly, BAG-1S also protected cells from the long-term growth-inhibitory effects of a range of other cellular stresses, including cytotoxic drugs, radiation, and hypoxia. The effects of BAG-1S on these agents were not as dramatic as those for heat shock. Although selected to result in equivalent levels of long-term growth inhibition, it is difficult, however, to directly compare these conditions in terms of stress that the cell receives. Moreover, it is not clear whether the same mechanism of
BAG-1 function in breast cancer cells

BAG-1-mediated protection will operate in each case. Regardless, the ability of BAG-1 to not simply delay cell death but also promote long-term growth has clear implications for its involvement in both the development of cancer and evolution of chemotherapy and radiation resistance clones.

It appears paradoxical that whereas this and other tissue culture studies demonstrate that BAG-1 prevents cell death, BAG-1 expression is associated with improved prognosis in some (32, 33, 36) but not all malignancies (41). This has been discussed previously (2, 33) and is not unique for BAG-1 because expression of Bcl-2 is also associated with improved prognosis in breast cancer. It is possible that inactivation of cell death pathways by increased expression of these proteins leads to a less aggressive tumor phenotype than other changes involving, for example, p53 mutation and/or altered growth factor receptor expression. Changes in BAG-1 expression may therefore still be important in the development of the tumors with which they are associated, although the tumors produced might have a less aggressive phenotype.

BAG-1 interferes with apoptosis induced by a wide range of factors and is considered a broadly active survival protein. However, it is important to note that in many of these studies, the effects of BAG-1 overexpression are rather modest and reflect a delay in the kinetics rather than prevention of cell death. Consistent with this, we have shown that BAG-1 overexpression has relatively modest effects on TRAIL-induced apoptosis. Like cytokine withdrawal and Fas-induced apoptosis, TRAIL-induced cell killing would not be expected to involve cell damage and/or activation of a stress response. Although additional studies are required in other cell types to determine whether this is a consistent finding, we hypothesize that stress-induced cell death pathways are the primary targets of BAG-1 in apoptosis control, although BAG-1 does impact on other cell death pathways. Consistent with this proposed primary role in controlling cellular responses to stress, translation of BAG-1S can occur by internal ribosome entry, a translation mechanism that is maintained after heat shock, in contrast to cap-dependent scanning (42).

How does BAG-1 function to suppress the effects of stress? BAG-1 proteins have been shown to interact with a wide range of cell targets that might contribute to the survival effects of BAG-1, including the antiapoptotic Bcl-2 protein, the Raf-1 kinase, chaperone molecules, and some growth factor receptors (1–5, 10, 12, 26). BAG-1L and BAG-1M also interact nonspecifically with DNA through basic amino acids that form part of the NH2-terminal NLS and prevent global suppression of transcription in heat-shocked cells (38, 43). However, all BAG-1 isoforms are equally active in suppressing growth inhibition, suggesting that DNA binding does not play a key role. We have also shown that BAG-1 enhances estrogen-dependent transcription in breast cancer cells. However, only BAG-1L stimulates estrogen receptor function, thereby discounting the possibility that the effects of overexpression were due to stimulation of estrogen-dependent cell survival pathways in MCF7 cells.

Our deletion mutagenesis demonstrated that both the NH2-terminal and COOH-terminal parts of BAG-1 were required for protection from stress. The COOH-terminal BAG domain interacts with both chaperone molecules and the Raf-1 kinase, both of which have pro-survival functions and might theoretically account for the effects of BAG-1. To dissect the role of these target molecules, we used a well-characterized point mutant of mouse BAG-1S that no longer interacted with chaperones but retained the ability to bind and activate Raf-1 (28). Although many functions of BAG-1 are suggested to be mediated by interaction with Hsc70/Hsp70, only regulation of androgen and glucocorticoid receptor function has been demonstrated to be dependent on chaperone binding (20, 44). Many other studies have shown a requirement of the BAG-1 COOH-terminal for survival function, however, these deletions would prevent binding to both chaperones and Raf-1. Indeed, the only study to dissect the role of these proteins suggested that BAG-1 activation of Raf-1 was negatively regulated by Hsp70, and mutants that failed to interact with chaperones were more active than the wild-type molecule in preventing heat shock-induced growth inhibition (28). In our cell system, the mutant defective in chaperone binding failed to prevent stress-induced growth inhibition, demonstrating for the first time that the prosurvival function of BAG-1S can be mediated by interaction with chaperones. The different results obtained in breast cancer cells and fibroblasts suggest that BAG-1 has multiple mechanisms of action to overcome growth-inhibitory effects of stress and that these may be cell type dependent.

Heat shock proteins appear to be key for BAG-1 function in breast cancer cells and, in addition to protein refolding, are involved in a range of other functions including ubiquitylation. Ubiquitin is a low molecular weight protein that is attached to substrates to target them for rapid turnover via the proteasome. A series of reactions catalyzed by E1, E2, and E3 enzymes culminate in the transfer of multiple ubiquitin moieties to specific protein substrates (45). In addition to chaperone binding, BAG-1 interacts with and regulates other components of the system, including the proteasome and E3 ligases, Siah and CHIP (5, 29, 30, 46, 47). BAG-1 interacts with the proteasome via its NH2-terminal parts, and although the requirement for specific residues for proteasome binding is not known, we have now demonstrated that a key conserved lysine residue is essential for BAG-1 function. Thus, our data are consistent with a model whereby BAG-1 exerts its protective effects, at least in part, by coordinating the function of chaperones and the ubiquitin/proteasome system to regulate the turnover of specific protein substrates (30). Presumably, the protein targets of BAG-1 regulated via turnover are key regulators of apoptosis and cell cycle.

It was initially surprising that the BAG-1 isoforms were functionally equivalent in protecting cells from effects of heat shock because they reside in different cellular compartments. However, we demonstrated that like chaperones and BAG-1M (38, 39), both endogenous and overexpressed BAG-1S relocated to the nucleus after heat shock. Therefore, although initially localized differentially, the BAG-1 iso-
forms may share a common site of action and substrates in the nucleus after heat shock. Surprisingly, endogenous BAG-1 and Hsp70/Hsp70 dissociated after heat shock, although the levels of BAG-1 and Hsc70 were not reduced in the nucleus of cells. We hypothesize that the BAG-1-chaperone interaction is regulated by stress signals and that a sufficiently severe “stress” leads to dissociation of BAG-1 and Hsp70/Hsp70, depriving cells of BAG-1-mediated survival functions and thereby contributing to cell death. By contrast, elevated BAG-1 levels increased BAG-1-chaperone complexes, and although they were decreased after heat shock, these complexes were still readily detectable. Thus, dynamic regulation of the BAG-1-Hsc70/Hsp70 interaction by stress signals and its perturbation by BAG-1 overexpression may play a critical role in controlling cellular response. The mechanism(s) controlling association and relocalization remains to be determined.

Nuclear localization of BAG-1S is unlikely to be dependent on chaperone binding because it is maintained after dissociation but may involve a putative bipartite NLS present within BAG-1S (17).

We also demonstrated that nonfunctional BAG-1 mutants interfered with wild-type BAG-1 function in a transdominant manner, consistent with the idea that these domains are important for protein-protein interaction. Interestingly, the growth of control MCF7 cells was consistently reduced by expression of nonfunctional BAG-1 mutants with an intact BAG domain, suggesting that activity of endogenous BAG-1 proteins is important for cell growth in breast cancer cells. BAG-1 mutants lacking the BAG domain also interfered with BAG-1 function in ZR-75-1 cells and decreased tumor size (25).

The profound effects on the response of cells to stress-induced growth inhibition suggest that BAG-1 overexpression confers a significant growth advantage to tumor cells, allowing them to survive within a stressful nutrient-deprived and/or hypoxic tumor environment. Our results also suggest that BAG-1 plays a significant role in determining resistance of tumor cells to therapies, such as cytotoxic drugs and radiation. We have also shown that BAG-1L stimulates estrogen receptor function, another key pathway determining growth and thereby contributing to cell death. By contrast, elevated BAG-1 levels increased BAG-1-chaperone complexes, and although they were decreased after heat shock, these complexes were still readily detectable. Thus, dynamic regulation of the BAG-1-Hsc70/Hsp70 interaction by stress signals and its perturbation by BAG-1 overexpression may play a critical role in controlling cellular response. The mechanism(s) controlling association and relocalization remains to be determined.

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