Regulation by heavy metals and temperature of the human BAG-3 gene, a modulator of Hsp70 activity

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Abstract In HeLa cells the expression of the BAG-3 gene, a member of the BAG family, is regulated by heavy metals and temperature, with kinetics of accumulation of mRNAs similar to Hsp70 and metallothioneins. Western blot assays performed with a polyclonal anti-BAG-3 antibody confirmed that higher levels of the protein were present in the cells following heat and metal exposure. By immunofluorescence techniques and cell fractionation assays we demonstrated that following stress BAG-3 protein concentrated in the rough endoplasmic reticulum and associated with the heavy membrane fraction. The role of BAG-3 protein during apoptosis and cellular stress is discussed.

Key words: BAG; Heavy metal; Cellular stress; Gene regulation

1. Introduction

Heat shock proteins (Hsps) have high structural similarities among various organisms, conserved throughout evolution [1]. Each family of mammalian Hsps – Hsp90, Hsp70, Hsp60 and the small Hsps – is composed of members expressed constitutively or following exposure to different agents, and/or targeted to different subcellular compartments [2]. The most abundant and best characterized group of Hsps is the Hsp70 family. The Hsps have a distinct domain structure with an ATP binding site located at the amino-terminal end and a carboxy-terminal domain with high affinity for misfolded polypeptides. Although both Hsp70 and Hsc70 (constitutive Hsp70 protein) proteins by themselves may exert some protective effects on denatured or misfolded peptides, they generally act in concert with other cellular components, which associate with Hsc70/Hsp70 [3]. Two classes of modulators of Hsp70 function have been identified: (i) chaperone enhancers, such as Hip, which favor the Hsp70 chaperone activity and Hsp40 (Hdj-1), which stimulates the Hsp70 ATPase activity [4]; (ii) chaperone inhibitors such as BAG-1, a member of the BAG protein family, initially characterized as a regulator of receptor activity and Bcl-2-dependent apoptosis, which associates 'in vivo' and 'in vitro' with Hsp70, inhibiting its protein refolding activity [5,6]. This evolutionarily conserved family of proteins contains a common BAG domain of roughly 50 amino acid residues near their COOH terminus, able to interact with the ATPase domain of Hsp70 or Hsc70 proteins, and differs consistently in their N-terminal regions [7].

In the present paper we report the novel finding that in HeLa cells exposure to heavy metals and heat positively regulates the expression of a member of the BAG gene family, BAG-3, together with Hsp70 and metallothionein (MT) genes. Stress increases the levels of the corresponding BAG-3 protein and causes its mobilization from the cytosol toward the rough endoplasmic reticulum (RER). Therefore the BAG-3 gene appears to play a dual role in preventing cell death and contributing to the cellular defense response to stress.

2. Materials and methods

2.1. Cell culture

HeLa cells were grown as described [8]. Before each experiment with metals, the medium was replaced with medium containing 0.1% fetal calf serum; after 20 h, the cells were exposed to 10 μM $CdCl_2$ or 250 μM $ZnCl_2$ (Sigma, St. Louis, MO, USA), or heat shock for the different times.

2.2. Northern blot analyses

10 μg of RNA from each sample was extracted [9] and analyzed by Northern blot [8]. The following probes were used: a partial clone of BAG-3 covering nucleotide sequence 1221–2034 (GenBank gi|14043023|ref|NM-004281.2) isolated from a cDNA library obtained from HeLa cells grown for 8 h in presence of 250 μM $ZnCl_2$; the complete hMT1e cDNA sequence [10]; the hHsp70 entire cDNA (gift of M.G. Santoro, Italy); the BAG-1M whole cDNA sequence (gift of J. Hohfeld, Germany); 18S rRNA oligonucleotide (BD Biosciences Clontech, Palo Alto, CA, USA). The probes were radiolabeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using a random primer-labeling system (Takara Shuzo, Shiga, Japan).

2.3. Antibodies

The BAG-3 polyclonal antiserum was obtained by injecting into rabbits the BAG-3 carboxy-terminal peptide NPSSMTDTPGNP-AAP_{COOH} conjugated to ovalbumin (Primm, Milan, Italy). Anti-calreticulin antibodies in immunofluorescence and Western blot experiments were a monoclonal antibody from StressGen Biotechnologies, Victoria, Canada, and a rabbit polyclonal antibody, respectively.

2.4. Western blots

100 μg of cell lysates in B-Buffer [150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA (pH 8) and 1% Triton X-100] were separated on 10% SDS–PAGE gels [11] and immunoblotted [12].

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Abbreviations: Hsp, heat shock protein; Hsc70, constitutive Hsp70 protein; HSE, heat shock elements; MT, metallothionein; MRE, metal regulatory elements; PLC-γ, phospholipase C-γ

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2.5. Immunofluorescence

Immunofluorescence procedures have been described in [11]. Secondary antibodies were from Sigma. Imaging was obtained with an Axiophot fluorescence microscope (Carl Zeiss Vision, München-Hallbergmoos, Germany).

2.6. Subcellular fractionation

Subcellular fractionation procedures have been described in [13].

3. Results

3.1. Regulation of the expression of BAG-3 gene by zinc and cadmium

Since the first partial BAG-3 cDNA clone was originally isolated from a library made from mRNA extracted from HeLa cells exposed to zinc for 8 h, we next examined by Northern blot experiments if the expression of this gene was regulated by heavy metals. Time-dependent kinetics of accumulation of a single BAG-3 mRNA species of 2800 nucleotides were found following exposure of the cells for different times to 250 μM $ZnCl_2$ (Fig. 1A) or 10 μM $CdCl_2$ (Fig. 1F). The two kinetics were slightly different: following exposure to zinc, BAG-3 RNA was mainly accumulated between 2 and 8 h (Fig. 1A, lanes 3–8) with a decrease at 10 h (Fig. 1A, lane 9); in cells grown in presence of cadmium the highest levels of

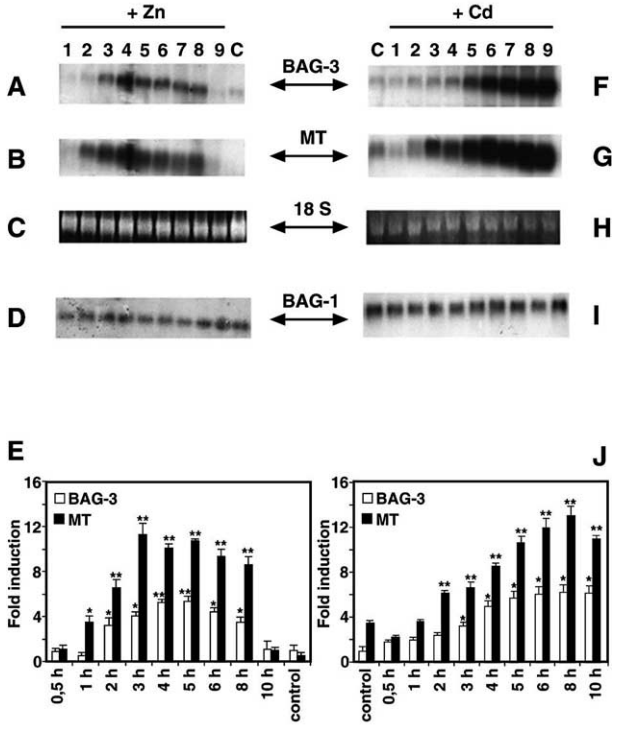

Fig. 1. Time-dependent kinetics of BAG-3 gene expression in HeLa cells exposed to zinc and cadmium. Northern blots of control HeLa cells (C) or treated with 250 μM $ZnCl_2$ (panels A–D) or 10 μM $CdCl_2$ (panels F–I) for different times (lane 1, 0.5 h; lane 2, 1 h; lane 3, 2 h; lane 4, 3 h; lane 5, 4 h; lane 6, 5 h; lane 7, 6 h; lane 8, 8 h; lane 9, 10 h. A,F: BAG-3 probe; B,G: hMT1e probe; C,H: 18S rRNA probe; D,I: BAG-1M probe. Quantitative analyses of the BAG-3 and MT mRNA levels were obtained by laser densitometry of the Northern autoradiograms of the zinc- (panel E) or cadmium- (panel J) exposed cells. mRNA levels are expressed as fold induction compared to unstimulated cells. Empty bars, BAG-3; solid bars, MT. Results are the means ± S.D. of three independent experiments. Statistical analyses were carried out by Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

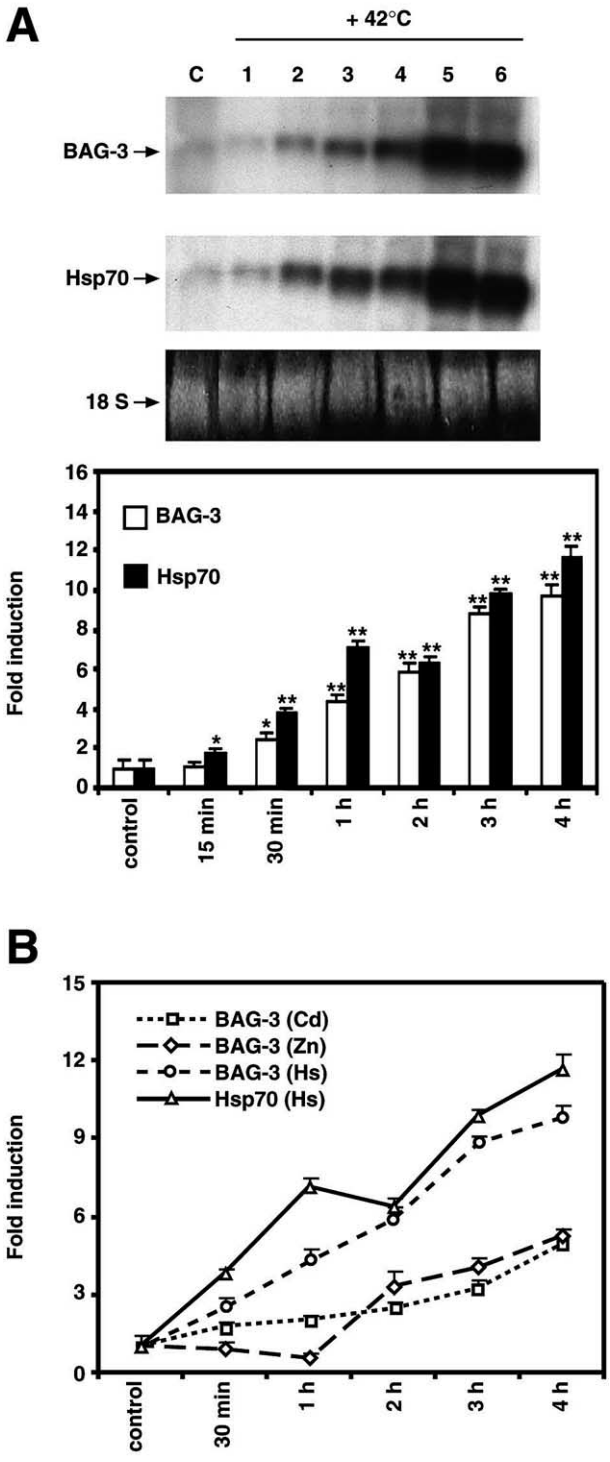

Fig. 2. Time-dependent kinetics of Hsp70 and BAG-3 gene expression in HeLa cells exposed to high temperature. A: Northern blots of control HeLa cells (C) or exposed for different times to 42°C (lane 1, 15 min; lane 2, 30 min; lane 3,1 h; lane 4, 2 h; lane 5, 3 h; lane 6, 4 h), using Hsp70, BAG-3 and 18S rRNA probes. B: Time-dependent accumulation of Hsp70 and BAG-3 mRNAs, following exposure to 42°C (HS), cadmium (Cd) and zinc (Zn). Quantitative analyses of the BAG-3 and Hsp70 mRNA levels were obtained by laser densitometry of the Northern autoradiograms, normalized for equal amounts of 18S rRNA in each lane. Data are expressed as fold induction compared to unstimulated cells. Results are the means ± S.D. of three independent experiments. Statistical analysis was carried out by Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

BAG-3 mRNA were detected from 4 to 10 h (Fig. 1F, lanes 5–9). No zinc- or cadmium-dependent regulation of the expression of the most characterized member of the BAG gene family, BAG-1, was detected (Fig. 1D,I). The kinetics of expression of the control metal-regulated genes, MTs, overlapped with the zinc- and cadmium-mediated responses of the BAG-3 gene (Fig. 1B,G). Quantitative analyses of BAG-3 and MT mRNA levels, normalized for identical levels of 18S ribosomal RNA per sample (Fig. 1C,H), are shown in Fig. 1E,J.

3.2. *Temperature is another regulator of the expression of BAG-3 gene*

Since BAG-3 protein is overexpressed and induced by heat stress in pancreatic cancer cell line [14], we investigated by Northern blot analyses the effect of the increase of the temperature on the expression of BAG-3 gene in HeLa cells. Both BAG-3 and Hsp70 mRNAs were highly accumulated in cells exposed to high temperature (42°C) with similar time-dependent kinetics (Fig. 2A), i.e. concentrations of both mRNAs increased between 30 min and 4 h (Fig. 2A lanes 2–6).

Comparison of the effects of metals (Fig. 1) and temperature (Fig. 2) on the extent and the kinetics of BAG-3 gene expression showed that metals are less potent regulators than temperature (Fig. 2B).

3.3. *The synthesis of BAG-3 protein increases following exposure to metals and heat*

We next investigated if all these stress conditions stimulate 'in vivo' the synthesis of BAG-3 protein. HeLa cells were grown for 8 h in presence of 10μM $CdCl_2$ or 250μM $ZnCl_2$ or exposed to a temperature of 42°C for 4 h. Western blot analyses of the protein lysates using a polyclonal anti-BAG-3 antibody revealed the presence of a polypeptide of 84 kDa relative molecular mass in control cells (Fig. 3, lanes 1, 3, 5) [13,14]. Its levels increased following exposure to cadmium, zinc and heat (Fig. 3, lanes 2, 4, 6). No differences were found in the levels of the control α-tubulin protein following stress.

3.4. *Exposure to metals and heat changes the subcellular localization of BAG-3 protein*

The intracellular localization of BAG-3 protein was next examined using indirect immunofluorescence microscopy. In control cells the signal appeared homogeneously distributed in the cytoplasm (Fig. 4A). When cells were exposed for 8 h to cadmium, zinc or to 42°C for 4 h the distribution of BAG-3 protein consistently changed, assuming a reticular, perinuclear localization on a cytosolic background (Fig. 4B–D). Double immunofluorescence experiments demonstrated that the signal for calreticulin, a protein resident in the RER, colocalizes with the one of BAG-3 protein in cadmium-treated cells (compare Fig. 4F with Fig. 4G) or exposed to zinc or heat (data not shown).

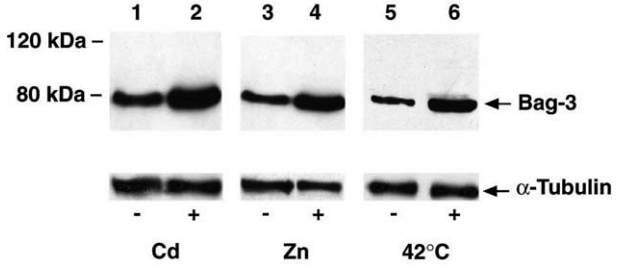

Fig. 3. In vivo synthesis of BAG-3 protein. Whole HeLa cell lysates, from controls (lanes 1, 3, 5) or cells exposed for 8 h to 10 μM $CdCl_2$ (lane 2) or 250 μM $ZnCl_2$ (lane 4), or for 4 h to 42°C (lane 6) were separated on 10% SDS–PAGE and analyzed with Western blot, using anti-BAG-3 polyclonal antibody or α-tubulin monoclonal antibody. Migrations of the protein molecular weight markers and BAG-3 or α-tubulin proteins are indicated on the left- and right-hand side, respectively.

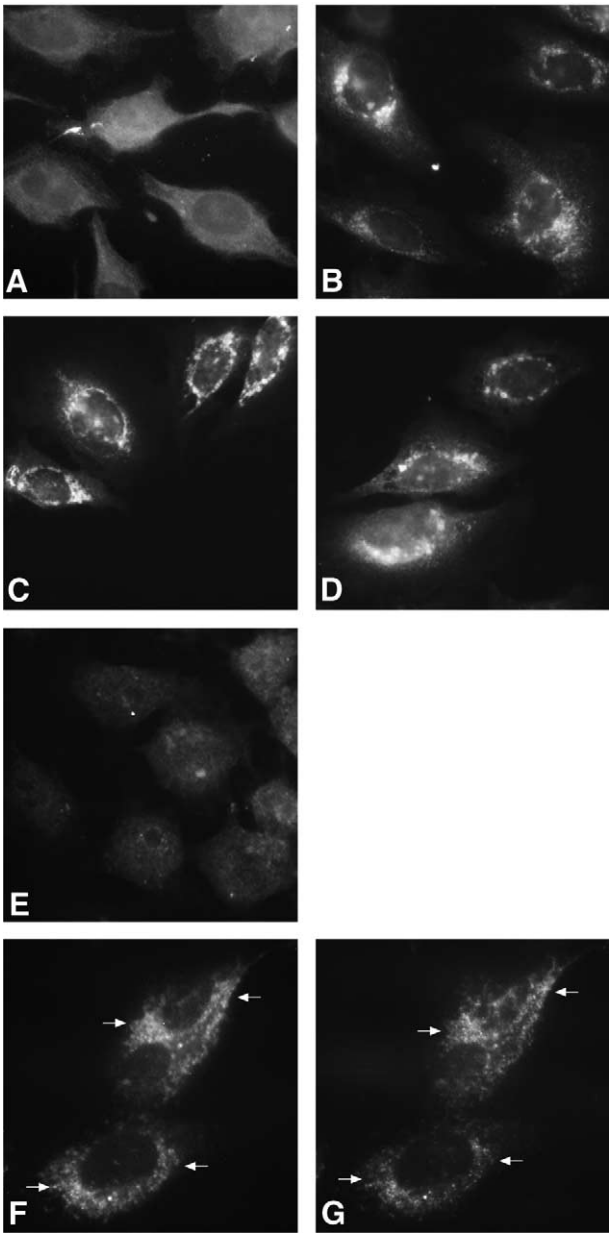

Fig. 4. Metals and heat change the intracellular distribution of BAG-3 protein. Control HeLa cells (panels A and E) or treated for 8 h with 10 μM $CdCl_2$ (panels B and F) or 250 μM $ZnCl_2$ (panel C) or for 4 h with heat (42°C) (panel D) were fixed, permeabilized and immunostained with the rabbit anti-BAG-3 polyclonal antibody (panels A–D and F) or with pre-immune serum (panel E) or anti-calreticulin monoclonal antibody (panel G). Arrows indicate points of major aggregation of BAG-3 (panel F) and calreticulin proteins (panel G).

3.5. Subcellular distribution of BAG-3 protein in control and cadmium-exposed HeLa cells

To confirm the previous results, cell fractionation experiments were performed. A quantitative analysis of such data is shown in Fig. 5E. In control cells BAG-3 protein was localized mainly in the cytosol (Fig. 5A, lane 1); a minor amount was associated with the light membrane fraction (Fig. 5A, lane 2). In cells grown in presence of cadmium an increase in the levels of BAG-3 protein was found in all fractions (Fig. 5A,B, compare lanes 1–5, and Fig. 5E). The enrichment of BAG-3 protein in the heavy membrane fractions agrees well with its intracellular localization in the endoplasmic reticulum (ER) in stressed cells, previously demonstrated by immunofluorescence microscopy (Fig. 4B–D). Some protein was also found associated with the nuclear fractions (Fig. 5B, lane 3). Control experiments made using anti-calreticulin antibodies showed that this protein, resident of the RER, was present in the heavy membrane and nuclear fractions (Fig. 5C,D, lanes 3 and 4). Since we were not able to detect by immunofluorescence microscopy a nuclear localization of BAG-3 protein in stressed cells (Fig. 4B–D and data not shown) we believe that this finding is due to contaminant perinuclear RER membranes associated with the nuclear envelope. Moreover, the high levels of BAG-3 protein found in the cytosolic fraction of stressed cells (Fig. 5B, lane 1) – which we did not fully detect in the previous immunofluorescence experiments – could also be explained by the cell fractionation procedure itself, in which a release from the membranes of part of the RER-complexed BAG-3 peptide could occur.

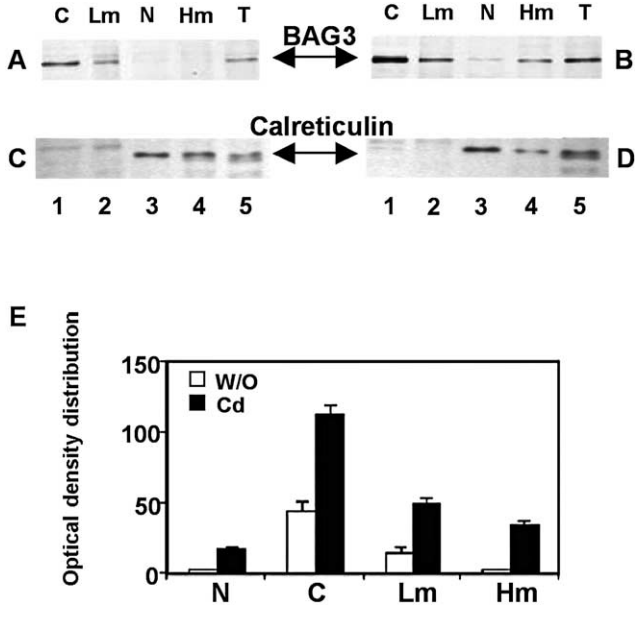

Fig. 5. Subcellular distribution of BAG-3 protein in control and cadmium-exposed HeLa cells. The distribution of BAG-3 protein was examined by subcellular fractionation followed by Western blot analyses with: anti-BAG-3 antibody (panels A,B); anti-calreticulin antibody (panels C,D). A,C: Control HeLa cells; B,D: cells treated for 8 h with 10 μM $CdCl_2$. T, total cell lysate; N, nuclear fraction after treatment with 0.5% NP40; C, cytoplasmic fraction; Lm, light membrane fraction; Hm, heavy membrane fraction. E: Quantitative analyses of the BAG-3 protein levels obtained by laser densitometry of Western blots. W/O: control cells (panel A). Cd: Cd-treated cells (panel B). Results are the means ± S.D. of three independent experiments.

4. Discussion

The family of BAG genes is highly conserved through evolution and can regulate different biochemical and cellular events, leading to cell death or cell differentiation or division [7]. All BAG proteins have at their COOH-terminus a BAG domain able to interact with and regulate the chaperone family of Hsp70 proteins, while the N-terminus is utilized to bridge specific proteins. We demonstrated that during metal- or temperature-dependent cell stress a coordinate response of three genes, Hsp70, MT and BAG-3, occurred. Similar results were also obtained in other cell lines, like Jurkat, neuroblastoma SK-N-SH, peripheral blood lymphocytes (data no shown). Further experiments are needed to establish if this regulation of the BAG-3 gene is mediated at the promoter level by characteristic *cis*-regulatory elements like the heat shock elements (HSEs) and the metal regulatory elements (MREs) [15,16] or by other sequences.

BAG-3 protein accumulates in the ER following stress: this organelle is the major point of integration of damage-sensing or pro-apoptotic stimuli. Several Bcl-2-binding proteins are present in it, and Bcl-2 has been reported to exert there part of its cytoprotective effect [17]. It is feasible that BAG-3 localization in the ER is due to its property to complex other proteins: no ER localization signal (i.e. KDEL) was found when analyzing the BAG-3 peptide sequence by the PSORT program (Expert Protein Analysis System server), while interactions with different cellular partners appear to play an important role in determining the function of BAG proteins [7]. In human melanoma cells A2058 exposed chronically to escalating quantities of the calcium entry blocker, CAI, the expression of BAG-3 gene (or CAIR-1) was increased and the protein was found to bind to phospholipase C-γ (PLC-γ) through a PXXP domain, forming a ternary complex with Hsp70/Hsc70 proteins [18]. We also confirmed the interaction between BAG-3 and PLC-γ proteins in control cells, but no relevant differences were detected following growth in presence of the metal (data not shown). BAG-3 protein also forms complexes with Bcl-2 polypeptide, resulting in an enhancement of its anti-apoptotic activity and a decrease in the apoptosis induced via Bax or Fas in the human epithelial cell line HeLa [13]. We found that BAG-3 specific antisense oligodeoxynucleotides were able to down-modulate BAG-3 protein levels and activate apoptosis in B-CLL (B chronic lymphocytic leukemia) cell cultures [12]. Since Hsp70, Hsp90 and Hsp27 can also block this process by acting at different levels of the death pathway [19–21], the effect of BAG-3 on cell death could be in part accounted for by its association with these molecules.

What is the role of BAG-3 gene in the stress response? In control HeLa cells BAG-3 and Hsc70 proteins interact, while in stressed cells BAG-3 forms complexes with both Hsc70 and Hsp70 proteins (data not shown). It has been shown that BAG-1M accelerated ATP-triggered substrate release from Hsc70/Hsp70, acting as a discharging factor for both these proteins [22]. This probably means that BAG-3, like BAG-1, could function as co-chaperone and modulate the folding activity of Hsc/Hsp70 chaperone machinery. Moreover, the association of BAG-3 with Hsp70 could influence the anti-apoptotic behavior of Hsp70 that is able to bind Apaf1 and inhibit the apoptosome assembly [2]. Quantitative and qualitative variations of such complex(es) could be relevant in

stress conditions, where also changes in the levels of BAG-3 and Hsps occur. This most probably happens via the association with other peptide partners via several cellular processes, aimed to protect cells from noxious stimuli which can lead up to apoptosis.

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