

Original Article

Different BAG-1 isoforms have distinct functions in modulating chemotherapeutic-induced apoptosis in breast cancer cells

Hong-yu LIU^{1,#}, Zhuo-min WANG^{2,#}, Yun BAI¹, Min WANG¹, Ying LI¹, Sen WEI¹, Qing-hua ZHOU^{1,*}, Jun CHEN^{1,*}

¹Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Lung Cancer Institute, Tianjin Medical University General Hospital, Tianjin 300052, China; ²The Key Laboratory of Lung Cancer Molecular Biology in Sichuan Province, West China Hospital, Sichuan University, Chengdu 610041, China

Aim: BAG-1 is a multifunctional anti-apoptotic gene with four isoforms, and different BAG-1 isoforms have different anti-apoptotic functions. In this study, we transfected BAG-1 isoforms into the human breast cancer cell lines Hs578T (ER negative) and MCF-7 (ER positive) to study their effect on apoptosis with or without estrogens.

Methods: The constructed recombinant expression vectors carrying individual BAG-1 isoforms was used to transfect human breast cancer cell lines Hs578T (ER negative) and MCF-7 (ER positive). After stable cell lines were made, a variety of apoptosis-inducing agents, including doxorubicin, docetaxel, and 5-FU, was used to treat these cell lines with or without estrogen to test the role of BAG-1. The mechanism by which BAG-1 affected the function of Bcl-2 was explored by using the cycloheximide chase assay.

Results: The BAG-1 p50 and p46 isoforms significantly enhanced the resistance to apoptosis in both cell lines according to flow cytometry analysis. BAG-1 p33 and p29 failed to protect the transfected cells from apoptosis. The cell viability assay showed that only BAG-1 p50, but not p46, p33, or p29, increased estrogen-dependent function in ER-positive cell line MCF-7. Only BAG-1 p50 dramatically increased its anti-apoptotic ability in the presence of estrogen, while estrogen has very little effect on the anti-apoptotic ability of other BAG-1 isoforms. In the detection of the expression of K-ras, Hsp70, cytochrome *c*, Raf-1, ER- α , and Bcl-2 in MCF-7 cells by Western blot, only Bcl-2 protein expression was significantly increased in MCF-7 cells transfected with BAG-1 p50 and p46, respectively. Furthermore, the cycloheximide chase assay indicated that the degradation of Bcl-2 protein was extended in the BAG-1 p50 and p46 transfected MCF-7 cells.

Conclusion: Distinct isoforms of BAG-1 have different anti-apoptotic functions in breast cancer cells, and that the BAG-1 p50 isoform can potentiate the role of estrogen in ER-positive breast cancer.

Keywords: BAG-1; breast cancer; estrogen; apoptosis

Acta Pharmacologica Sinica (2009) 30: 235–241; doi: 10.1038/aps.2008.21; published online 19th January 2009

Introduction

Breast cancer is the most common cancer to affect women. In 2007, it is estimated that about 240,510 new cases of breast cancer were diagnosed in the United States. Estrogen plays an important role in the development of breast cancer. Breast cell growth — both normal and abnormal — is stimulated by the presence of estrogen. Estrogen is mediated by estrogen receptors through estrogen response

elements (ERE) and functions as a ligand-dependent transcription factor. Hormonal therapy is a very effective treatment against breast cancer with hormone receptor-positive patients, which blocks the ability of estrogen to turn on and stimulate the growth of breast cancer cells. Therefore, it is very important to identify the factors that influence ER function and understand the roles that estrogen may play in the development of breast cancer.

BAG-1 is a recently identified multifunctional anti-apoptotic protein that binds to Bcl-2^[1] and RAF-1 serine/threonine kinase^[2]. BAG-1 cooperates with Bcl-2 to inhibit apoptosis triggered by a variety of apoptotic agents. To date, four isoforms of BAG-1 protein have been reported, each having different anti-apoptotic functions in different cell lines. BAG-1 has been observed to bind to proteins

[#] Hong-yu LIU and Zhuo-min WANG contributed equally to this paper.

* Co-correspondence to Qing-hua ZHOU and Jun CHEN.
E-mail zhouqh1016@yahoo.com.cn (Qing-hua ZHOU);
huntercj2004@yahoo.com (Jun CHEN).

Received 2008-11-01 Accepted 2008-12-02

from four different subcellular compartments: cytosolic domains of tyrosine kinase HGF/PDGF receptors on outer cell membranes, Bcl-2 on inner cell membranes, cytosolic Hsps, RAF-1 and nuclear hormone receptors. However, the significance of these associations in regulating apoptosis is poorly understood. The interaction between BAG-1 and Bcl-2 likely plays an essential role in BAG-1-regulated apoptosis, although other BAG-1-interactive proteins may also be important. Recently, studies have also shown that forced expression of BAG-1 in cervical cancer cells leads to increased Bcl-2 production, while downregulation of BAG-1 expression by antisense BAG-1 results in the opposite^[3, 4]. BAG-1 potentiates the ability of Bcl-2 to inhibit apoptosis, but the exact mechanism by which BAG-1 co-expresses with Bcl-2 is currently unknown. BAG-1 may affect the function of Bcl-2 by modulating its expression either at the transcriptional or post-translation level. The increased expression of Bcl-2 in cells transfected with BAG-1 suggests that intact BAG-1 function may be required to inhibit Bcl-2 protein degradation. In addition, a retrospective study was recently conducted by using 185 paraffin-embedded breast tumor tissues. The study indicated that a majority (86.0%) of breast cancers over-expressed BAG-1 and that most (85.5%) of those positive for BAG-1 staining were cytoplasmic. Interestingly, statistical analysis indicated that BAG-1 expression was correlated with that of Bcl-2, p53, estrogen receptor (ER) and progesterone receptors^[5]. Furthermore, interaction of BAG-1 with estrogen receptor (ER) stimulates the transcriptional activity of ER α and ER β ^[6]. It is possible that BAG-1 may affect the development of breast cancer through modulating the function of ER, and therefore it is very important to address the function of BAG-1 on estrogen receptors in breast cancer.

In this study, we used the constructed recombinant expression vectors carrying individual BAG-1 isoforms to transfect human breast cancer cell lines Hs578T (ER negative) and MCF-7 (ER positive). After stable cell lines were made, we used a variety of apoptosis-inducing agents, including doxorubicin, docetaxel, and 5-FU, to treat these cell lines in the presence or absence of estrogen to test the role of BAG-1. Furthermore, we also explored the mechanism by which BAG-1 affected the function of Bcl-2 by using the cycloheximide chase assay.

Materials and methods

Construction of the recombinant plasmids carrying the four BAG-1 isoforms

All the structures of human BAG-1 isoforms have been

described, and plasmids containing BAG-1 p50, p46, p33 and p29 were constructed as described previously^[3, 7].

Cell culture and transfection

Human breast cancer cell lines MCF-7 and Hs578T were purchased from Cell Line Service. MCF-7 cells were grown in Minimum Essential Medium (MEM, Gibco), and Hs578T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with penicillin/streptomycin (180 IU/mL) in 10% (*v/v*) heat-inactivated fetal calf serum (FCS) and 1% (*v/v*) *L*-glutamine. They were incubated in a humidified atmosphere with 5% CO₂ in air at 37 °C. DNA transfection was carried out using the Effectene Transfection Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells (5×10^4 cells/well) were seeded into 6-well plates and incubated at 37 °C in 5% CO₂ overnight. Next day, 0.5 μ g of plasmid DNA was mixed with Effectene Transfection Reagent and added onto the cell monolayer. The cells were then further incubated for an additional 48 h. Stable transfectants were selected in selective medium containing 0.6 mg/mL G418 (Gibco BRL) and maintained in the same medium for use.

Cell viability assay

Cells were seeded (5×10^3 cells/well) into 96-well plates and incubated for 24 h, and then the cells were treated with 10 pmol/L 17- β estradiol for 24 h. Cell viability was defined as the fold increase of BAG-1 distinct isoform-transfected cells compared with the control cells in the presence of 17- β estradiol by using the CellTiter 96[®] Aqueous One Solution Reagent assay. Control transfectant was used as the standard. Each experiment was performed in triplicate and repeated at least three times.

To reduce endogenous estrogen-like activity, phenol red-free DMEM medium (Gibco BRL) supplemented with 5% dextran-coated charcoal-stripped FBS (Hyclone, Logan, UT, USA) (estrogen-deprived medium) was used.

Flow cytometry analysis of apoptotic cells

To determine the effect of distinct isoforms of BAG-1 protein on apoptosis, 1×10^5 cells/well were seeded into 6-well plates and incubated for 24 h, and then the cells were treated with doxorubicin (0.5 μ mol/L), docetaxel (0.1 μ mol/L), and 5-FU (150 μ mol/L) for 48 h. Cells were stained using the Annexin V-FITC Apoptosis Analysis Kit (PharMingen) and subjected to a FACStar Plus flow cytometer (Becton Dickinson) to sort out the Annexin V-FITC stained apoptotic cells. Data was analyzed with WIMDI 2.8 software. The apoptotic cells (M1) were calculated as the

percentage of apoptotic cells in the treated cell population minus the percentage of apoptotic cells in the untreated control cell population.

Western blot analysis

Cellular protein extraction and Western blot analyses were performed as previously described^[3]. Briefly, 20 μ g of protein was fractionated using 10%–12% SDS-PAGE and transferred onto Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membranes under semidry conditions. Immunodetection was performed using the ECL system (Amersham Pharmacia Biotech). Monoclonal antibodies against K-ras, Hsp70, cytochrome *c*, Raf-1, ER- α , Bcl-2, BAG-1 and β -actin were purchased from Santa Cruz.

Cycloheximide chase assay

To determine the change of Bcl-2 protein stability in BAG-1 transfected cell lines, the cycloheximide chase assay was performed. In brief, 100 μ g/mL of cycloheximide (Biomol, Plymouth Meeting, PA) was added to about 60% semi-confluence cells on 100-mm plates for 24 h. An equal number of cells was harvested at various time points. The amount of Bcl-2 at each time point was analyzed by Western blot with an anti-Bcl-2 antibody.

Statistical analysis

For cell flow cytometry, each experiment was performed in triplicate and repeated three times. For the cell viability assay, each experiment was performed in triplicate and repeated at least three times. Data were analyzed using the Student's *t*-test. A $P \leq 0.05$ was considered statistically significant.

Results and discussion

Expression of distinct isoforms of BAG-1 in MCF-7 and Hs578T cells

BAG-1 has four isoforms (p50, p46, p33 and p29), which have distinct N-termini^[8]. The largest isoform, p50, contains a nuclear localization sequence in the N-terminal extension and resides in the nucleus, while isoform p46 contains only a small part of the nuclear localization sequence and is found in both the nucleus and cytoplasm. The most abundant isoform is p33 and the smallest isoform is p29, both of which are without any nuclear localization sequence, and are found predominantly in the cytoplasm. Since the four isoforms are generated from a single mRNA transcript^[7], translational regulation is thought to play a major role in the control of their expression. The native BAG-1 mRNA

carries four protein translation start codons, and each start codon lacks the perfect Kozak sequence^[7]. It has been shown that initiation of the synthesis of the four isoforms of BAG-1 occurs by an alternative mechanism^[7]. As previously described^[3], we constructed recombinant plasmids carrying individual BAG-1 cDNA, with each start codon surrounded by a perfect Kozak sequence. We used the human breast cancer cell lines Hs578T (ER negative) and MCF-7 (ER positive), which both express low levels of BAG-1. After stable transfection and colony selection, cellular proteins were extracted to verify the expression of the distinct isoforms of BAG-1 in the transfectants by Western blot. Transfection with the BAG-1 isoforms p50, p46, p33, p29 generated the desired isoforms because of the existence of the perfect Kozak sequence (Figure 1).

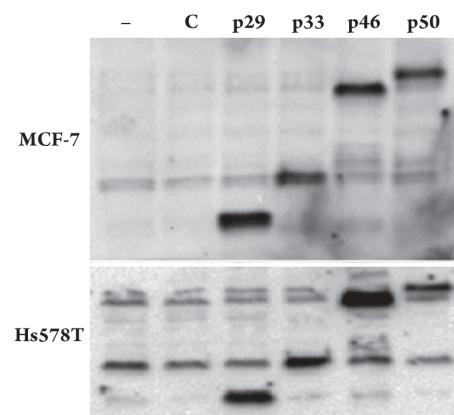


Figure 1. Expression of the full length and the deletion-mutant BAG-1 proteins after transfection in MCF-7 and Hs578T cells. All BAG-1 protein structures were described previously. The vector control plasmid pCR3.1 and the plasmids containing full-length BAG-1, p50K, p46K, p33K, and p29K cDNA were stably transfected into MCF-7 and Hs578T cells. The MCF-7 and Hs578T cells expressing different cDNA were lysed in protein lysis buffer and 20 μ g of protein was analyzed by Western blot with 12% SDS-PAGE. -: primary cells, c: control cells.

BAG-1 p50 and p46, but not p33 and p29, inhibit apoptosis in breast cancer cell lines

BAG-1 is known to protect cells from a wide range of apoptotic stimuli^[9, 10]. Different BAG-1 isoforms have been reported to have different effects on heat shock protein function^[11] and to possess different transcriptional activities^[12, 13]. Further studies have indicated that distinct BAG-1 isoforms have different anti-apoptotic functions in human cervical carcinoma cells. BAG-1 p50 and p46 isoforms enhanced the resistance to apoptosis in transfected cells, while BAG-1 p29 failed to protect the transfected

cells from apoptosis^[3]. It is still unclear whether the over-expression of BAG-1 alone is sufficient to inhibit apoptosis, or whether its anti-apoptotic function requires the presence of Bcl-2, as well as whether the functions of different BAG-1 isoforms in different tissues are different.

To examine the role of BAG-1 isoforms in anti-apoptotic activity in breast cancer cell lines, BAG-1-transfected Hs578T and MCF-7 cells were treated with a variety of apoptosis-inducing agents, including doxorubicin (0.5 $\mu\text{mol/L}$), docetaxel (0.1 $\mu\text{mol/L}$), and 5-FU (150 $\mu\text{mol/L}$). After 48-h incubation, cell apoptosis was assessed with Annexin-V FITC by cell flow cytometry. The FITC-positive apoptotic cells were expressed as the percentage of apoptotic cells over the total number of treated cells minus the percentage of apoptotic cells of the untreated cells, as shown in Figure 2. Compared to the NEO-transfected control cells, the MCF-7 cells transfected with BAG-1 p50 and p46 exhibited significant resistance to apoptosis when treated with doxorubicin (32.77%, 28.98% vs 58.76%, $P<0.05$), docetaxel (31.68%, 32.77% vs 56.38%, $P<0.05$) and 5-FU (47.37%, 40.47% vs 63%, $P<0.05$). Hs578T cells transfected with BAG-1 p50 and p46 also showed a similar increased resistance to apoptosis when treated with doxorubicin (17.7%, 14.8% vs 38.98%, $P<0.05$), docetaxel (18.7%, 23.48% vs 41.68%, $P<0.05$) and 5-FU (26.08%, 23% vs 39.77%, $P<0.05$). Both cell lines transfected with BAG-1 p33 and p29 showed no significant change in their sensitivity to apoptosis when compared to the NEO-transfected control cells treated with doxorubicin (MCF-7: 50.68%, 56.74% vs 58.76%, $P>0.05$; Hs578T: 31.1%, 34.8% vs 38.98%, $P>0.05$), docetaxel (MCF-7: 51.76%, 54.76% vs 56.38%, $P>0.05$; Hs578T: 30.01%, 39.8% vs 41.68%, $P>0.05$) and 5-FU (MCF-7: 57.64%, 54.77% vs 63%, $P>0.05$; Hs578T: 38.8%, 36.8% vs 39.77%, $P>0.05$). These results indicate that distinct BAG-1 isoforms have different anti-apoptotic functions in breast cancer cell lines MCF-7 and Hs578T. BAG-1 p50 and p46 exhibited a significant degree of anti-apoptotic activity ($P<0.05$), while BAG-1 p33 and p29 had little effect. These results were very similar to those of our previous studies on a human cervical cancer cell line^[3].

BAG-1 p50, but not p46, p33, and p29, increases MCF-7 cell viability in the presence of estrogen

Estrogens play an important role in development of breast cancer and stimulate the proliferation and survival of breast cancer cells. The action of estrogens is mediated by estrogen receptors (ERs). Adjuvant hormonal therapies such as tamoxifen counter the actions of estrogens and reduce the probability of death and recurrence in those with estrogen

receptor positive cancer. BAG-1 is a multifunctional anti-apoptotic protein, and our previous studies indicated that BAG-1 expression was correlated with ER in breast cancer tissues^[5]. To test the role of estrogen with BAG-1 isoforms, we added 10 pmol/L 17- β estradiol into MCF-7 and Hs578T cell lines transfected with different BAG-1 isoforms, and then performed cell viability assays after 24 h. The results, shown in Figure 2, indicate that in the presence of estrogen, the cell viability in MCF-7 cells transfected with BAG-1 p50 (3.18 fold), but not with p46 (1.614 fold), p33 (1.085 fold) and p29 (0.9985 fold), was significantly increased compared to the control cells, while there was no significant change in Hs578T cells transfected with distinct BAG-1 isoforms (Figure 3). This result indicated that only BAG-1 p50 potentiated the estrogen-dependent signal pathway and enhanced the function of estrogen in the ER-positive cell line MCF-7. Furthermore, to test the role of estrogen with stimuli, MCF-7 cells transfected with different BAG-1 isoforms were treated with chemotherapeutic agents in the presence of 10 pmol/L 17- β estradiol. As shown in Figure 4, compared with the NEO-transfected control cells, the BAG-1 p50 transfected MCF-7 cells exhibited

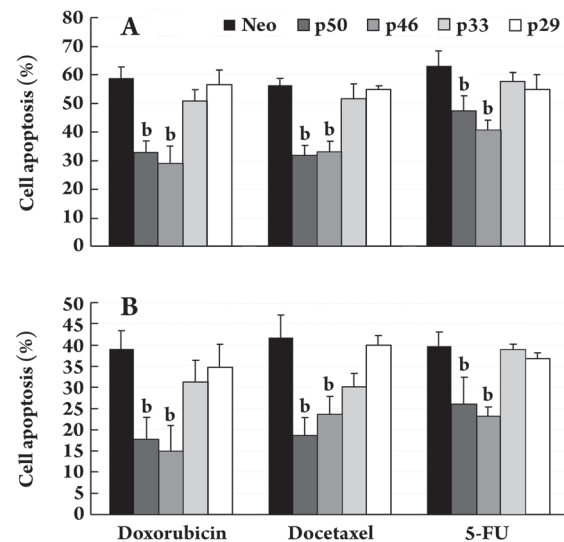


Figure 2. Effect of the BAG-1 isoform transfection on MCF-7 (A) and Hs578T (B) cell death induced by chemotherapy drugs. Cells (5×10^3 cells/well) were seeded into 96-well plates and incubated for 48 h, and then the cells were treated with different drugs (0.5 $\mu\text{mol/L}$ doxorubicin, 0.1 $\mu\text{mol/L}$ docetaxel, 150 $\mu\text{mol/L}$ 5-FU) for 48 h. Cell apoptosis represents the percentage of apoptotic cells in the treated cell population minus the percentage of apoptotic cells in the untreated control cell population as determined using the Annexin V-FITC Apoptosis Analysis Kit (PharMingen) and a FACStar plus flow cytometer (Becton Dickinson) to sort out the Annexin V-FITC stained apoptotic cells. $n=3$. Mean \pm SD. ^b $P<0.05$ vs NEO control.

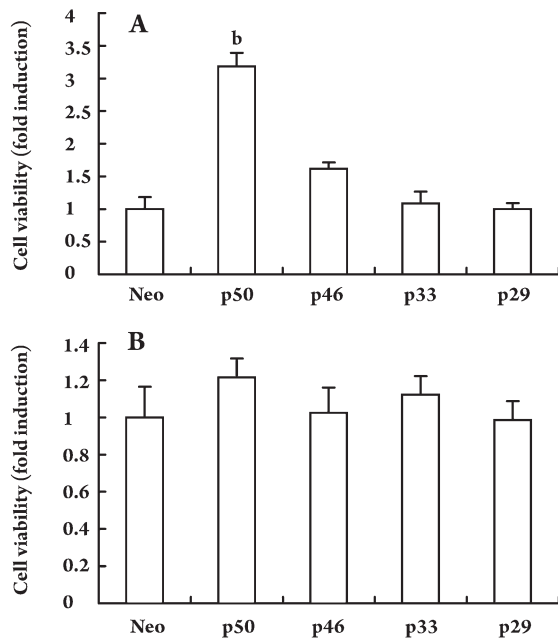


Figure 3. Effect of BAG-1 isoform transfection on MCF-7 (A) and Hs578T (B) cell growth induced by 17- β estradiol. Cells (5×10^3 cells/well) were seeded into 96-well plates and incubated for 24 h, and then the cells were treated with 10 pmol/L 17- β estradiol for 24 h. Cell viability represents the fold increase of BAG-1 distinct isoform-transfected cells compared with the control cells in the presence of 17- β estradiol using the CellTiter 96[®] Aqueous One Solution Reagent assay. The control transfectant was used as the standard. $n=3$. Mean \pm SD. ^b $P<0.05$ vs NEO control.

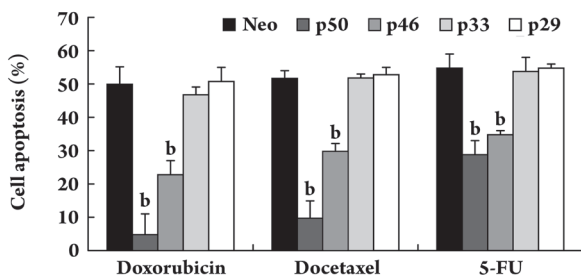


Figure 4. Effect of the BAG-1 isoform transfection on MCF-7 cell death induced by chemotherapy drugs in the presence of 10 pmol/L 17- β estradiol. Cells (5×10^3 cells/well) were seeded into 96-well plates and incubated for 24 h, and then the cells were treated with different drugs (0.5 μ mol/L doxorubicin, 0.1 μ mol/L docetaxel, 150 μ mol/L 5-FU) for 48 h. $n=3$. Mean \pm SD. ^b $P<0.05$ vs NEO control.

the strongest resistance to apoptosis in the presence of estrogen when treated with doxorubicin (4.77% vs 49.9%, $P<0.05$), docetaxel (9.68% vs 51.67%, $P<0.05$) and 5-FU (28.76% vs 54.76%, $P<0.05$), which indicated that its role in resistance to apoptosis in the presence of estrogen was

much stronger than that without estrogen. Compared to the NEO-transfected control cells, cells transfected with BAG-1 p46 had significantly increased resistance to apoptosis in the presence of estrogen when treated with doxorubicin (22.76% vs 49.9%, $P<0.05$), docetaxel (29.76% vs 51.67%, $P<0.05$) and 5-FU 34.77% vs 54.76%, $P<0.05$), but the role of BAG-1 in the presence of estrogen was very similar to that without estrogen. The MCF-7 cells transfected with BAG-1 p33 and p29, even in the presence of estrogen, showed no change in their sensitivity to apoptosis compared to the NEO-transfected control cells when treated with doxorubicin (46.74%, 50.74% vs 49.9%, $P<0.05$), docetaxel (51.76%, 52.76% vs 51.67%, $P<0.05$) and 5-FU (53.76%, 54.76% vs 54.76%, $P<0.05$). These results indicate that only BAG-1 p50 can potentiate the role of estrogen in the ER-positive cell line MCF-7, and are similar to findings of other reports that BAG-1 p50 can interact with ER and increase estrogen-dependent transcription^[6].

The different anti-apoptotic function of BAG-1 isoforms may be due to their structural differences. All four BAG-1 isoforms have a common C-terminus, which contains the BAG domain^[14] that interacts with Hsp70^[15,16], Bcl-2^[17] and hepatocyte growth factor (HGF) receptor^[9]. BAG-1 p50 has the complete nuclear localization sequence, whereas BAG-1 p46 has only a partial nuclear localization sequence (NLS), which explains the common and occasional nuclear expression of BAG-1 p50 and BAG-1 p46, respectively. BAG-1 p46 is produced mainly as a cytosolic protein, and BAG-1 p33 and BAG-1 p29 are always produced in the cytosol^[7]. The exact mechanism by which BAG-1 participates in anti-apoptotic activity is unknown. As described in our previous reports, the differential anti-apoptotic function of different BAG-1 isoforms suggests that the N-terminus of the protein is important for its function. Previous reports have indicated that the N-terminus was important for BAG-1 to bind to hormone receptors^[18,19], transcription factors such as c-Fos^[20] and certain DNA promoters, such as the CMV promoter^[21]. There is a hexapeptide repeat region in BAG-1 N-terminus. BAG-1 p50 and p46 share a complete sequence, BAG-1 p33 shares a partial sequence, and p29 lacks this region altogether^[22]. Although the function of this hexapeptide repeat region is unclear, it is tempting to speculate that this hexapeptide repeat region is implicated in the anti-apoptotic function of BAG-1, since BAG-1 p50 and p46 with the complete hexapeptide repeat have strong anti-apoptotic function. Deletion of this region renders the protein highly unstable^[22]. The increased anti-apoptotic ability of BAG-1 p50 in the presence of estrogen in ER-positive cells may be due to the interaction between BAG-1 p50 and ER, given

that BAG-1 p50 is the only isoform that directly interacts with ER and potentiates estrogen-dependent transcription^[6].

Increased expression of Bcl-2 in MCF-7 and Hs578T cells transfected with BAG-1 p50 and p46, but not with p33 and p29

To investigate why the overexpression of BAG-1 isoforms lead to the differential resistance to apoptosis induced by different chemotherapeutic agents in transfected cells, we examined the expression of a group of apoptotic regulating proteins—K-ras, Hsp70, cytochrome *c*, Raf-1, ER- α , and Bcl-2—in MCF-7 cells stably transfected with the BAG-1 isoforms by Western blot analysis. Compared with the control cells transfected with NEO, transfection with BAG-1 p50 and p46, but not p33 and p29, led to the increased expression of Bcl-2. The expression of all other apoptotic proteins, including K-ras, Hsp70, cytochrome *c*, Raf-1 and ER- α , remained no significant change (Figure 5). β -actin was used as an internal

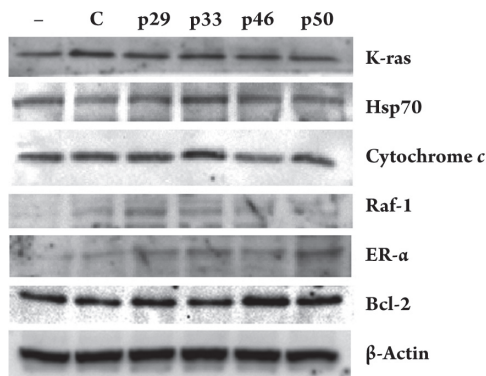


Figure 5. Expression of apoptosis and drug resistance-associated proteins in MCF-7 cells stably transfected with the vector control plasmid pCR3.1(C) and plasmids containing the four BAG-1 isoforms' respective cDNA. Western blot analysis was performed using 20 μ g of protein from different cells. β -actin was used as an internal control.

control for protein quantity in each experiment. Our previous studies demonstrated that the increased expression of Bcl-2 was likely due to decreased Bcl-2 protein degradation, and not to the increased mRNA transcription, since Bcl-2 mRNA remained essentially unchanged after transfection with native BAG-1 and the BAG-1 isoforms compared to the NEO-transfected control cells by Northern blotting^[3]. Next, the pulse-chase assay was employed to analyze the effect of BAG-1 isoforms on the translation level Bcl-2 protein and its protein stability. As shown in Figure 6A-B, the stability of Bcl-2 protein in the BAG-1 high expression cell line MDA-MB231, but not in the BAG-1 low expression cell line MCF-7, was extended. Furthermore, the stability of Bcl-2 in MCF-7 cells transfected with BAG-1 p50 and p46, but not p33, p29 and the NEO control, was increased. The results indicated that the BAG-1 p50 and p46 isoforms affected Bcl-2 protein stability and increased the level of Bcl-2 protein (Figure 6C).

Except for Bcl-2, transfection of BAG-1 does not change the expression of its related proteins, such as Hsp70, K-ras, cytochrome *c*, ER- α , and Raf-1. Bcl-2 protein expression was increased in cells transfected with BAG-1 p50 and p46, but not p33 and p29 or NEO, which is consistent with our previous data. Since our previous study showed that the expression of Bcl-2 mRNA did not change after transfection with BAG-1, we proposed that the forced expression of certain BAG-1 isoforms might be decreased by Bcl-2 protein degradation. In this study, the protein stability of Bcl-2 in MCF-7 cells transfected with BAG-1 p50 and p46, but not p33, p29 and the Neo control, was confirmed to be increased by the pulse-chase assay. All BAG-1 isoforms share a common C-terminus that binds to Hsp, Bcl-2 and the proteasome. The increased expression of Bcl-2 in cells transfected with BAG-1 p50 and p46, but not BAG-1 p33 and p29, suggests that intact BAG-1 function is required for the inhibition of Bcl-2 protein degradation mediated by BAG-1.

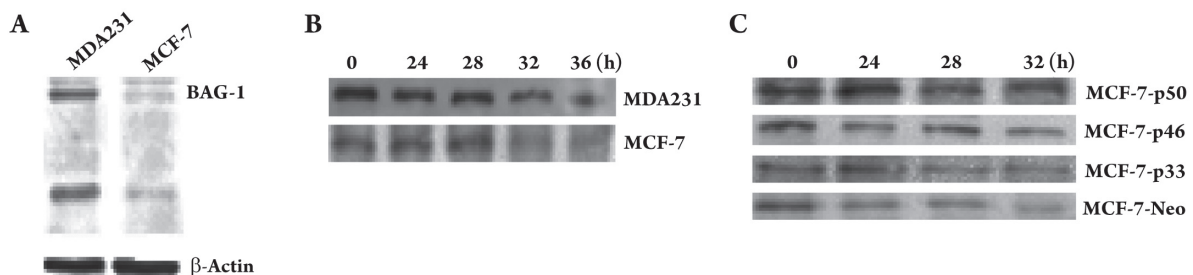


Figure 6. (A) Expression of BAG-1 in MDA-MB231 and MCF-7. (B) The stability of Bcl-2 protein by the cycloheximide chase assay in MDA-MB231 and MCF-7 cells. Equal numbers of cells were analyzed by Western blotting. (C) The stability of the Bcl-2 protein by the cycloheximide chase assay in MCF-7 cells transfected with different BAG-1 isoforms. Equal numbers of cells were analyzed by Western blotting.

BAG-1 has been reported to cooperate with Bcl-2 to inhibit apoptosis^[1].

In summary, our data demonstrate that distinct BAG-1 isoforms have different anti-apoptotic functions in breast cancer cell lines and that the BAG-1 p50 and p46 isoforms enhance resistance to apoptosis. BAG-1 p50 is the only isoform that can potentiate estrogen-dependent ER function. The anti-apoptotic function of BAG-1 isoforms may be correlated to increased Bcl-2 expression, which may be mediated through decreased Bcl-2 protein degradation.

Acknowledgement

This work was supported by grants from the National Natural Science Foundation of China (No 30500221 to Jun CHEN; No 30500496 to Hong-yu LIU). We also acknowledge the National Key Technology Research and Development Program of China (No 2006BAI02A00, Qing-hua ZHOU).

Author contribution

Hong-yu LIU performed research, analyzed data, and wrote the paper; Zhuo-min WANG performed research, analyzed data; Yun BAI, Min WANG, Ying LI and Sen WEI performed research; Qing-hua ZHOU designed the study, analyzed the data and wrote the paper; Jun CHEN designed the study, performed research, analyzed data, and wrote the manuscript.

References

- 1 Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, *et al*. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell* 1995; 80: 279–84.
- 2 Wang HG, Takayama S, Rapp UR, Reed JC. Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc Natl Acad Sci USA* 1996; 93: 7063–8.
- 3 Chen J, Xiong J, Liu H, Chernenko G, Tang SC. Distinct BAG-1 isoforms have different anti-apoptotic functions in BAG-1-transfected C33A human cervical carcinoma cell line. *Oncogene* 2002; 21: 7050–9.
- 4 Xiong J, Chen J, Chernenko G, Beck J, Liu H, Pater A, *et al*. Antisense BAG-1 sensitizes HeLa cells to apoptosis by multiple pathways. *Biochem Biophys Res Commun* 2003; 312: 585–91.
- 5 Tang SC, Beck J, Murphy S, Chernenko G, Robb D, Watson P, *et al*. BAG-1 expression correlates with Bcl-2, p53, differentiation, estrogen and progesterone receptors in invasive breast carcinoma. *Breast Cancer Res Treat* 2004; 84: 203–13.
- 6 Cutress RI, Townsend PA, Sharp A, Maison A, Wood L, Lee R, *et al*. The nuclear BAG-1 isoform, BAG-1L, enhances oestrogen-dependent transcription. *Oncogene* 2003; 22: 4973–82.
- 7 Yang X, Chernenko G, Hao Y, Ding Z, Pater MM, Pater A, *et al*. Human BAG-1/RAP46 protein is generated as four isoforms by alternative translation initiation and overexpressed in cancer cells. *Oncogene* 1998; 17: 981–9.
- 8 Cato AC, Mink S. BAG-1 family of cochaperones in the modulation of nuclear receptor action. *J Steroid Biochem Mol Biol* 2001; 78: 379–88.
- 9 Bardelli A, Longati P, Albero D, Goruppi S, Schneider C, Ponzetto C, *et al*. HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. *Embo J* 1996; 15: 6205–12.
- 10 Clevenger CV, Thickman K, Ngo W, Chang WP, Takayama S, Reed JC. Role of Bag-1 in the survival and proliferation of the cytokine-dependent lymphocyte lines, Ba/F3 and Nb2. *Mol Endocrinol* 1997; 11: 608–18.
- 11 Luders J, Demand J, Papp O, Hohfeld J. Distinct isoforms of the cofactor BAG-1 differentially affect Hsc70 chaperone function. *J Biol Chem* 2000; 275: 14817–23.
- 12 Niyaz Y, Zeiner M, Gehring U. Transcriptional activation by the human Hsp70-associating protein Hap50. *J Cell Sci* 2001; 114 (Pt 10): 1839–45.
- 13 Zeiner M, Niyaz Y, Gehring U. The hsp70-associating protein Hap46 binds to DNA and stimulates transcription. *Proc Natl Acad Sci USA* 1999; 96: 10194–9.
- 14 Sondermann H, Scheufler C, Schneider C, Hohfeld J, Hartl FU, Moarefi I. Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. *Science* 2001; 291: 1553–7.
- 15 Takayama S, Bimston DN, Matsuzawa S, Freeman BC, Aime-Sempe C, Xie Z, *et al*. BAG-1 modulates the chaperone activity of Hsp70/Hsc70. *EMBO J* 1997; 16: 4887–96.
- 16 Takayama S, Xie Z, Reed JC. An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *J Biol Chem* 1999; 274: 781–6.
- 17 Antoku K, Maser RS, Scully WJ Jr, Delach SM, Johnson DE. Isolation of Bcl-2 binding proteins that exhibit homology with BAG-1 and suppressor of death domains protein. *Biochem Biophys Res Commun* 2001; 286: 1003–10.
- 18 Froesch BA, Takayama S, Reed JC. BAG-1L protein enhances androgen receptor function. *J Biol Chem* 1998; 273: 11660–6.
- 19 Kullmann M, Schneikert J, Moll J, Heck S, Zeiner M, Gehring U, *et al*. RAP46 is a negative regulator of glucocorticoid receptor action and hormone-induced apoptosis. *J Biol Chem* 1998; 273: 14620–5.
- 20 Zeiner M, Gebauer M, Gehring U. Mammalian protein RAP46: an interaction partner and modulator of 70 kDa heat shock proteins. *EMBO J* 1997; 16: 5483–90.
- 21 Takahashi N, Sasaki R, Takahashi J, Takayama S, Reed JC, Andoh T. BAG-1M, an isoform of Bcl-2-interacting protein BAG-1, enhances gene expression driven by CMV promoter. *Biochem Biophys Res Commun* 2001; 286: 807–14.
- 22 Luders J, Demand J, Hohfeld J. The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *J Biol Chem* 2000; 275: 4613–7.