

Distinct BAG-1 isoforms have different anti-apoptotic functions in BAG-1-transfected C33A human cervical carcinoma cell line

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BAG-1 protein can be expressed as four isoforms of 50, 46, 33 and 29 kDa with different subcellular localizations, which may have different functions in anti-apoptosis, but the exact mechanism remains unclear. We constructed BAG-1 full length and deletion mutated plasmids in a pCR3.1 vector and established stable transfections of BAG-1 isoforms in low BAG-1 expressing C33A cells. Treatment of the transfected cells with cisplatin, staurosporine, paclitaxel and doxorubicine showed that BAG-1 p50, p46 and p33 isoforms enhanced the resistance to apoptosis. BAG-1 p50, p46 and p33 exhibited different degrees of apoptosis inhibition in the transfected cells and BAG-1 p46 isoform had the most pronounced effect on anti-apoptosis. BAG-1 p29 failed to protect the transfected cells from apoptosis. Resistance to apoptosis by BAG-1 isoforms was correlated with decreased caspase-3 activation. We also detected the expression of Bax, Bak, p53, Bcl-2, Bcl-X_L, AIF and MRP1 by Western blots. Bcl-2 protein expression was significantly increased in p50, p46 and p33 transfected cells, while the expression of Bax, Bak, p53, Bcl-X_L and MRP1 was essentially unchanged. These *in vitro* results suggest that distinct isoforms of BAG-1 have different anti-apoptotic functions and their functions may be correlated to increased Bcl-2 expression.

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Introduction

BAG-1 is a multifunctional anti-apoptotic protein that binds to a variety of cellular proteins and affects their functions. Interaction of BAG-1 with Bcl-2, HGF and PDGF receptors and a variety of nuclear hormone receptors leads to the modulation of their functions and inhibition of apoptosis (Takayama *et al.*, 1995; Zeiner and Gehring, 1995; Bardelli *et al.*, 1996; Kullmann *et al.*, 1998; Liu *et al.*, 1998). We have observed that the interaction of BAG-1 with vitamin D

receptor (VDR) resulted in inhibition of vitamin D-induced apoptosis in cancer cells (Witcher *et al.*, 2001), while the interaction of BAG-1 with retinoic acid receptor (RAR) suppressed retinoic acid-induced apoptosis (Liu *et al.*, 1998). In addition, binding of BAG-1 to Raf-1 serine/threonine kinase significantly increases its kinase activities (Wang *et al.*, 1996). Furthermore, binding of BAG-1 to heat shock proteins inhibits the refolding of denatured proteins and protects cells from heat shock-induced apoptosis (Zeiner *et al.*, 1997; Hohfeld and Jentsch, 1997; Nollen *et al.*, 2001). Under the heat shock-induced stress, BAG-1 bound to DNA directly and enhanced expression of specific reporter gene constructs (Zeiner *et al.*, 1999). In addition, BAG-1 increased transcription of reporter gene constructs with different promoters in various cell types in the absence of thermal stress (Niyaz *et al.*, 2001). Over-expression of BAG-1 renders cells less susceptible to damage induced by heat stress.

The exact mechanism by which BAG-1 inhibits apoptosis is currently unknown. The existence of the highly conserved BAG-1 family proteins across species suggests that BAG-1 plays an important role in embryogenesis, stress response, apoptosis, growth inhibition and oncogenesis (Takayama *et al.*, 1999; Sondermann *et al.*, 2001; Antoku *et al.*, 2001; Adachi *et al.*, 1998; Clevenger *et al.*, 1997), although to date, BAG-1 has not been observed to inhibit apoptosis by itself. Forced expression of Bcl-2 and BAG-1 blocked nerve growth factor withdrawal-induced apoptosis, activation of caspases, and reactive oxygen species generation, suggesting that it functions upstream of caspases (Shulz *et al.*, 1997). We have recently noted that the resistance to apoptosis correlated with the increased BAG-1 expression and reduced caspase-3 activation in human cervical multidrug-resistant cells (Ding *et al.*, 2000a,b). BAG-1 may participate in anti-apoptosis through its interaction with Hsp70/Hsc70 by inhibiting Hsp-mediated apoptosis. In addition, we have successfully cloned *bag-1* promoter and reported the upregulation of BAG-1 expression by mutant p53. Our observation provided a novel mechanism that links the BAG-1-regulated apoptosis with the p53-dependent cell cycle control (Yang *et al.*, 1999a) during oncogenesis.

We demonstrated that BAG-1 protein was expressed as three major isoforms, designated p50 (BAG-IL), p46

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(BAG-1M), and p33 (BAG-1S) and one minor isoform, p29, with apparent molecular masses of 50, 46, 33 and 29 kDa, respectively (Yang *et al.*, 1998a). We provided convincing evidence that the four protein products were expressed by alternative initiation from the four different start codons through a leaky scanning mechanism. Furthermore, we showed that the distinct isoforms of BAG-1 have different subcellular localizations, suggesting that they perform distinct cellular functions. Characterization of BAG-1 RNA and protein revealed that BAG-1 was over-expressed in human cervical, lung and breast cancer cell lines (Zapata *et al.*, 1998), and that the expression of BAG-1 increased progressively after immortalization and transformation of human endocervical cells (HEN) by human papilloma virus type 16 (HPV-16) and cigarette smoke condensate (CSC) Yang *et al.*, 1998b). We demonstrated that both BAG-1 RNA and protein were over-expressed in human breast cancer cell lines and breast cancer tissues (Yang *et al.*, 1999b). More importantly differential expression of BAG-1 isoforms were noted in our study, which showed that over-production of *cytoplasmic* BAG-1 isoforms p46 and p33 may be involved in the pathogenesis of human breast cancer. We recently conducted a retrospective pilot study of BAG-1 expression in patients with invasive breast cancer (Tang *et al.*, 1999). Our study demonstrated for the first time that BAG-1 is widely over-expressed in breast cancer patients. In addition, we observed two patterns of BAG-1 expression, cytoplasmic and nuclear, as was noted in breast cancer cell lines. BAG-1 expression, especially nuclear BAG-1 expression was associated with a shorter disease-free and overall survival. Consistent with our observation, Turner *et al.* (2001) noted a positive correlation between the expression of *cytoplasmic* BAG-1 and longer distance metastases-free and overall survival in patients with early stage breast cancer. The association of the *cytoplasmic* BAG-1 expression and a better prognosis was also observed in our recent study in patients with non-small cell lung cancer (Rorke *et al.*, 2001).

Differential expression of BAG-1 was also recently reported in prostate, colon and leukemia cell lines (Brimmell *et al.*, 1999). Distinct isoforms of BAG-1 was noted to affect Hsc70 chaperone function differently (Luders *et al.*, 2000b). BAG-1 p46 (BAG-1M) was found to inhibit the heat shock-70-mediated refolding of the non-native polypeptide substrates, while BAG-1 p33 (BAG-1S) stimulated Hsc70 chaperone activity. The different amino-termini of the distinct BAG-1 isoforms appeared to determine the outcome of an Hsc70-mediated folding event. In addition, BAG-1 p50 and p46 activate gene transcription in the absence and presence of thymostress respectively (Niyaz *et al.*, 2001; Zeiner *et al.*, 1999). Using *in situ* hybridization and immunohistochemical staining, Crocoll *et al.* (2000) recently observed isoform-specific expression of BAG-1 in mouse development. While BAG-1 p50 (BAG-1L)-specific antibody showed ubiquitous staining, as early as day 10.5 post-coitum, there was progressive restriction during subsequent stages of

embryogenesis. On the contrary, the expression of BAG-1 p46 (BAG-1M) and p33 (BAG-1S) was only noted in the mouse myocardium in the early developmental stage. Stage and site-specific expression of BAG-1 isoforms appear to be important during mouse development.

Taken together, these data strongly suggest that the four isoforms of BAG-1 protein play a differential role in apoptosis and oncogenesis of human cancers. Furthermore, different BAG-1 protein isoforms may be used as biomarkers in the prognosis and treatment of many malignancies. To test this hypothesis, we have constructed plasmids carrying individual BAG-1 isoforms (p50, p46, p33 and p29), transfected the recombinant plasmids into a human cervical carcinoma cell lines, C33A, which expresses low level of BAG-1 and examined the role played by each BAG-1 isoform in anti-apoptosis. In addition, changes in other apoptotic proteins and the activation of caspase-3 were also examined. Our study showed for the first time that transfection of BAG-1 alone led to increased resistance to apoptosis. In addition, distinct BAG-1 isoforms have different anti-apoptotic functions in the transfected cells. Furthermore, increased resistance to apoptosis by BAG-1 correlated with the decreased caspase-3 activation and the increased expression of Bcl-2, but not with the other apoptotic proteins.

Results

Expression of distinct forms of BAG-1 in C33A cells

The native Bag-1 mRNA carries four protein translation start codons, each lacking the perfect Kozak sequences (Yang *et al.*, 1998a). Forced expression of BAG-1 by transfection with the native BAG-1 cDNA would therefore generate all four BAG-1 isoforms through a leaky scanning mechanism, making the functional assessment of individual BAG-1 isoforms difficult. To produce individual BAG-1 isoforms (p50, p46, p33, p29), we constructed the recombinant plasmids carrying the individual BAG-1 cDNA, with each start codon surrounded by a perfect Kozak sequence. The structures and functional domains of the individual BAG-1 isoforms are summarized in Figure 1. We used the human cervical carcinoma cell line, C33A, that expresses low levels of BAG-1, since most cancer cell lines we tested have moderate or high expression of BAG-1 mRNA or proteins. After stable transfection was established by growth in selection media, cellular proteins were extracted to verify the expression of the distinct isoforms of BAG-1 in the transfectants by Western blots. Transfection with the native BAG-1 full-length cDNA without Kozak sequence gave rise to the four isoforms by alternative translation initiation through a leaking scanning mechanism (Figure 2a), (Yang *et al.*, 1998a). Transfection with the BAG-1 isoforms p50, p46, p33, p29 generated the desired isoforms because of the existence of the perfect Kozak sequence (Figure 2b).

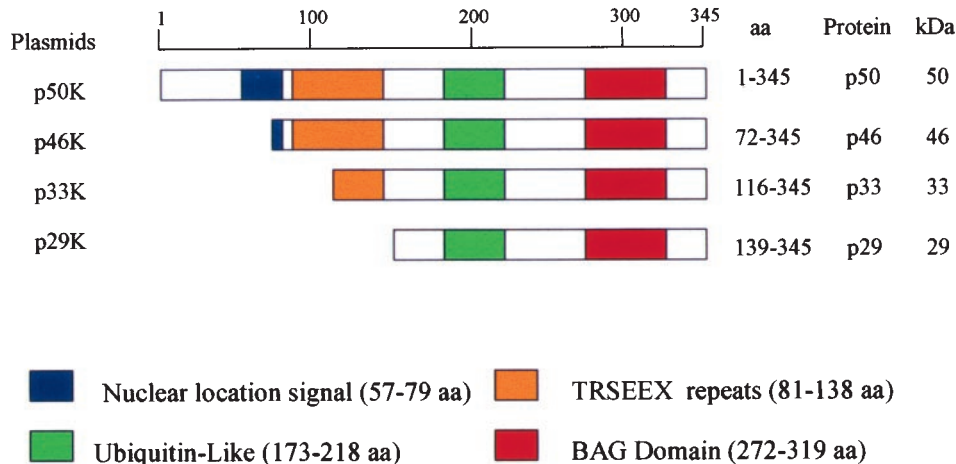


Figure 1 Structure of human BAG-1 isoforms. To prevent the alternative translation initiation from downstream start codons, a Kozak sequence was constructed in frame 5' to each start codon by PCR before inserting the product into the eukaryotic expression plasmids indicated on the left. Amino acid (aa) positions are shown on the top and right. The apparent molecular weights in kDa of protein products are shown on the right. The p50, p46, p33 and p29 isoforms are distinguished by the lengths of their amino termini. All isoforms share a ubiquitin-like domain and carboxyl-terminal domain that is sufficient for Hsc70 binding. p50 and p46 isoforms share the complete, and p33 isoform has a part of TRSEEX' hexapeptide repeats (Luders, 2000a). BAG-1 p50 has the complete, while BAG-1 p46 possesses a part of the nuclear location signal (NLS)

BAG-1 p50, p46, and p33 but not p29 inhibited apoptosis in C33A cells

To examine the role of distinct isoforms of BAG-1 in anti-apoptosis, BAG-1-transfected C33A cells were treated with a variety of apoptosis-inducing agents including cisplatin (10.0 μ M), staurosporine (1.0 μ M), paclitaxel (4.0 μ M), and doxorubicine (2.0 μ M), the optimal drug concentration that induced 25–50% apoptosis in the NEO control cells. After 48 h incubation, cell viabilities were assessed by the CellTiter96[®] Aqueous One Solution Reagent (Promega). The viable cells after drug treatment are expressed as a percentage of the viable cells over the total number of the treated cells on the histogram, as illustrated in Figure 3. Compared to the NEO-transfected control cells, the C33A cells transfected with BAG-1 p46 exhibited the strongest resistance to apoptosis when treated with cisplatin (77.87 vs 39.00%, $P < 0.05$), staurosporine (82.31 vs 36.64%, $P < 0.05$), paclitaxel (80.44 vs 45.76%, $P < 0.05$) and doxorubicine (65.24 vs 33.49%, $P < 0.05$). Compared to the NEO-transfected control, cells transfected with native BAG-1 and the BAG-1 p50 had the similar increased resistance to apoptosis when treated with cisplatin (67.18, 66.50 vs 39.00%, $P < 0.05$), staurosporine (73.58, 74.41 vs 36.64%, $P < 0.05$), paclitaxel (71.04, 74.23 vs 45.76%, $P < 0.05$) and doxorubicine (58.33, 60.13 vs 33.49%, $P < 0.05$). The C33A cells transfected with BAG-1 p29 showed no change in the sensitivities to apoptosis compared to the NEO-transfected control cells when treated with cisplatin (40.39 vs 39.00%, $P = 0.3223$), staurosporine (36.93 vs 36.64%, $P = 0.7978$), paclitaxel (44.67 vs 45.76%, $P = 0.2666$) and doxorubicine (32.77 vs 33.49%, $P = 0.4849$). Interestingly, the C33A cells transfected with BAG-1 p33 had a significantly

increased resistance to apoptosis induced by cisplatin (58.47 vs 39.00%, $P < 0.05$) and staurosporine (67.16 vs 36.64%, $P < 0.05$), but not by paclitaxel (46.61 vs 45.76%, $P = 0.3994$) and doxorubicine (34.10 vs 33.49%, $P = 0.5320$). Similar proportional inhibition of apoptosis was observed with each apoptotic agent when different drug concentrations were used (result not shown).

We further examined the apoptotic cells by Annexin-V FITC cell flow cytometry. The FITC-positive apoptotic cells were expressed as the percentage of apoptotic cells over the total number of the treated cells minus the percentage of apoptotic cells of the untreated cells, as shown as histogram in Figure 4. Constant with the cell viability study, our experiments demonstrated that, compared with the NEO-transfected control cells, the C33A cells transfected with BAG-1 p46 exhibited strongest resistance to apoptosis when treated with cisplatin, (23.67% apoptotic cells vs 68.86%, $P < 0.05$), staurosporine (16.42 vs 49.16%, $P < 0.05$), paclitaxel (22.80 vs 59.24%, $P < 0.05$) and doxorubicine (33.93 vs 69.67%, $P < 0.05$). Compared to the NEO-transfected control, cells transfected with the native BAG-1 and the BAG-1 p50 had the similar increased resistance to apoptosis when treated with cisplatin (35.59, 38.32% vs 68.86%, $P < 0.05$), staurosporine (21.67, 23.06 vs 49.16%, $P < 0.05$), paclitaxel (39.97, 40.19 vs 59.24%, $P < 0.05$) and doxorubicine (45.31, 45.20 vs 69.67%, $P < 0.05$). The C33A cells transfected with BAG-1 p29 showed no change in the sensitivities to apoptosis compared to the NEO-transfected control cells when treated with cisplatin (64.71 vs 68.86%, $P = 0.1558$), staurosporine (46.75 vs 49.16%, $P = 0.4566$), paclitaxel (57.91 vs 59.24%, $P = 0.2709$) and doxorubicine (66.95 vs 69.67%, $P = 0.1196$). The C33A cells transfected with BAG-1

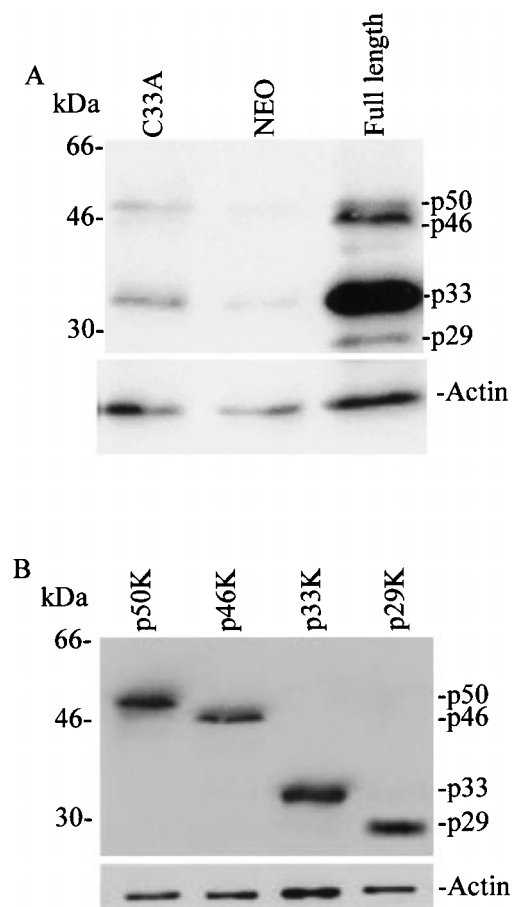


Figure 2 Expression of the full length and the deletion-mutant BAG-1 protein after transfection in C33A. All BAG-1 protein structures are described in Figure 1. The vector control plasmid pCR3.1 and the plasmids containing BAG-1 full length, p50K, p46K, p33K, p29K cDNA were stably transfected into C33A cells. The C33A cells expressing different cDNA were lysed in protein lysis buffer and 10 μ g protein were analysed by Western blot with 12% SDS-PAGE. (a) BAG-1 protein expression in C33A cells and in C33A cells transfected with pCR3.1 vector and BAG-1 full length plasmids, respectively. (b) Expression of four BAG-1 isoforms in C33A cells

p33 had a significantly increased resistance to apoptosis induced by cisplatin (46.21 vs 68.86%, $P < 0.05$) and staurosporine (32.22 vs 49.16%, $P < 0.05$), but not by paclitaxel (56.51 vs 59.24%, $P = 0.1131$) and doxorubicin (67.63 vs 69.67%, $P = 0.1289$).

We also studied the apoptotic effect of cisplatin on each BAG-1 isoform-transfected C33A cells by TUNEL assays (Figure 5a,b). Compared to the NEO-transfected control cells (56.75% apoptotic cells), the cells transfected with BAG-1 p46 had the lowest number of apoptotic cells (18.17%, $P = 0.000011$), followed by the native BAG-1 (28.94%, $P = 0.000123$), BAG-1 p50 (32.21%, $P = 0.000125$) and BAG-1 p33 (35.22%, $P = 0.000459$) transfected cells, when treated with cisplatin. The BAG-1 p29 transfected cells had the similar number of apoptotic cells (52.26%, $P = 0.1571$) compared to the control.

Increased expression of Bcl-2 in C33A cells transfected with the native BAG-1, BAG-1 p50, p46 and p33 but not with p29

To investigate why overexpression of BAG-1 isoforms lead to the differential resistance to apoptosis induced by different chemotherapeutic agents in C33A cells, we examined by Western blot analysis the expression of a group of apoptotic regulating proteins, Bcl-2, Bax, Bak, Bcl-X_L, AIF (apoptosis inducing factor) and p53 in the C33A cells stably transfected with the BAG-1 isoforms. Compared with the control cells transfected with the NEO, transfection with the native BAG-1, BAG-1 p50, p46 and p33, but not with the p29, led to the increased expression of Bcl-2, as measured by densitometry. Fold increase was calculated by dividing all the ratios (Bcl-2/ β -actin) for each of the BAG-1 isoforms by the NEO control (native BAG-1, 2.03%; p50, 1.43%; p46, 2.33%; p33, 1.99%; and p29, 0.88%, respectively). The expression of all other apoptotic proteins including Bax, Bak, Bcl-X_L, p53 and AIF remained unchanged (Figure 6). β -actin was used as internal control for protein quantity in each experiment. Similar increase in Bcl-2 protein expression was seen in C33A cells transfected with the BAG-1 isoforms and treated with apoptotic agents such as cisplatin and staurosporine (data not shown). Northern blot analysis further showed that the increase expression of Bcl-2 was not due to the increased mRNA transcription, since the Bcl-2 mRNA remained essentially unchanged after transfection with the native BAG-1 and the BAG-1 isoforms, compared to the NEO-transfected control cells (Figure 7).

Decreased caspase-3 activation in C33A cells transfected with the native BAG-1, BAG-1 p50 and p46, but not with BAG-1 p33 and p29

Studies have shown that activation of effector caspases, especially caspase-3, is one of the most important events in apoptosis. Caspase-3 is required for DNA fragmentation (Jänicke *et al.*, 1998). We examined, by Western blot and quantification by densitometry, the activation of caspase-3 after transfection of the C33A cells with BAG-1 isoforms and treatment with cisplatin. Our results showed that caspase-3 activation was unchanged in C33A cells transfected with the native BAG-1, BAG-1 p50, p46, p33 and p29, compared to the NEO-transfected control cells. However, after treatment with cisplatin for 48 h, there was 5–10 times reduction in the amount of the 17-kDa caspase-3 in cells transfected with the native BAG-1, BAG-1 p50 and p46, but not with the BAG-1 p33 or p29 (Figure 8).

Discussion

BAG-1 is a multifunctional anti-apoptotic protein recently discovered in a search for Bcl-2 interacting proteins using a protein interaction cloning technique.

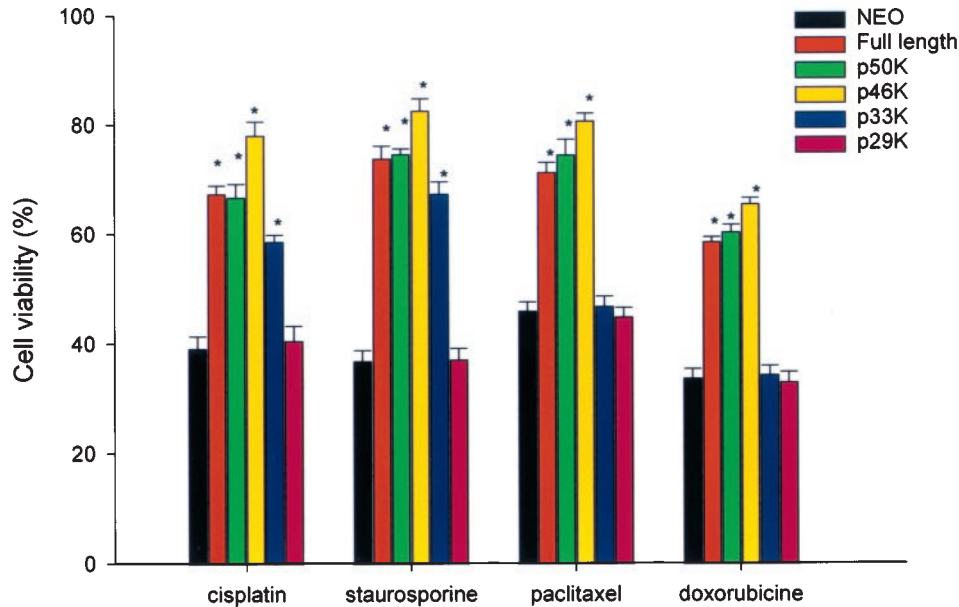


Figure 3 Effect of the native BAG-1 and BAG-1 isoforms transfection on C33A cell death induced by chemotherapy drugs. 5×10^3 cells/well were seeded into 96-well plates and incubated for 24 h, then cells were treated with different drugs ($1.0 \mu\text{M}$ staurosporine, $10 \mu\text{M}$ cisplatin, $4 \mu\text{M}$ paclitaxel, and $2 \mu\text{M}$ doxorubicine) for 48 h. Cell viability represents the percentage of viable cells over the total treated cells using CellTiter 96[®] Aqueous One Solution Reagent assay. *Denotes the cell viability that is statistically different from that of the cells transfected with NEO control ($P < 0.05$). The results represent three independent experiments

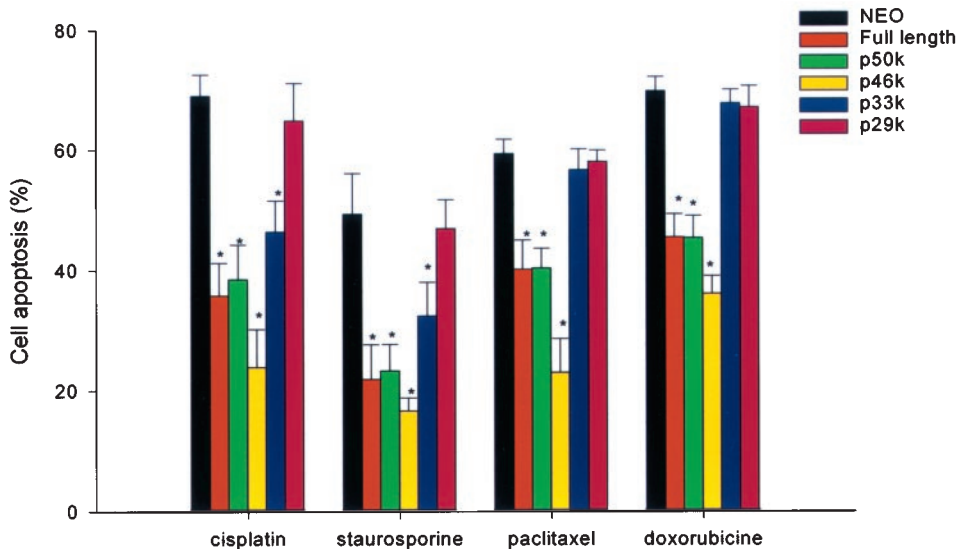


Figure 4 The effect of BAG-1 full length and distinct isoforms on apoptosis induced by chemotherapy drugs in C33A cells using Annexin V-FITC flow cytometry assays. Stably transfected NEO control and full length and different isoforms of BAG-1 over-expressing C33A cells were untreated or treated with different drugs ($1.0 \mu\text{M}$ staurosporine, $10 \mu\text{M}$ cisplatin, $4 \mu\text{M}$ paclitaxel, and $2 \mu\text{M}$ doxorubicine) for 48 h. *Denotes the cell apoptosis that is statistically different from that of the cells transfected with NEO control ($P < 0.05$). The results represent three independent experiments

BAG-1 enhanced the anti-apoptotic function of Bcl-2 when the two genes were co-transfected into a human lymphoid cell line, Jurkat cells (Takayama *et al.*, 1995). Co-transfection of BAG-1 and Bcl-2 enabled hybridoma cells to survive when treated with excess thymidine to arrest cell cycle (Terada *et al.*, 1997). Although BAG-1 was shown to collaborate with Bcl-2 to activate

RAF-1 locally in mitochondria and other Bcl-2-containing membranes in a Ras-independent manner (Wang *et al.*, 1996), the exact mechanism by which BAG-1 interacts with Bcl-2 to inhibit apoptosis is currently unknown. It is also unclear whether the over-expression of BAG-1 alone is sufficient to suppress apoptosis, and whether its anti-apoptotic function

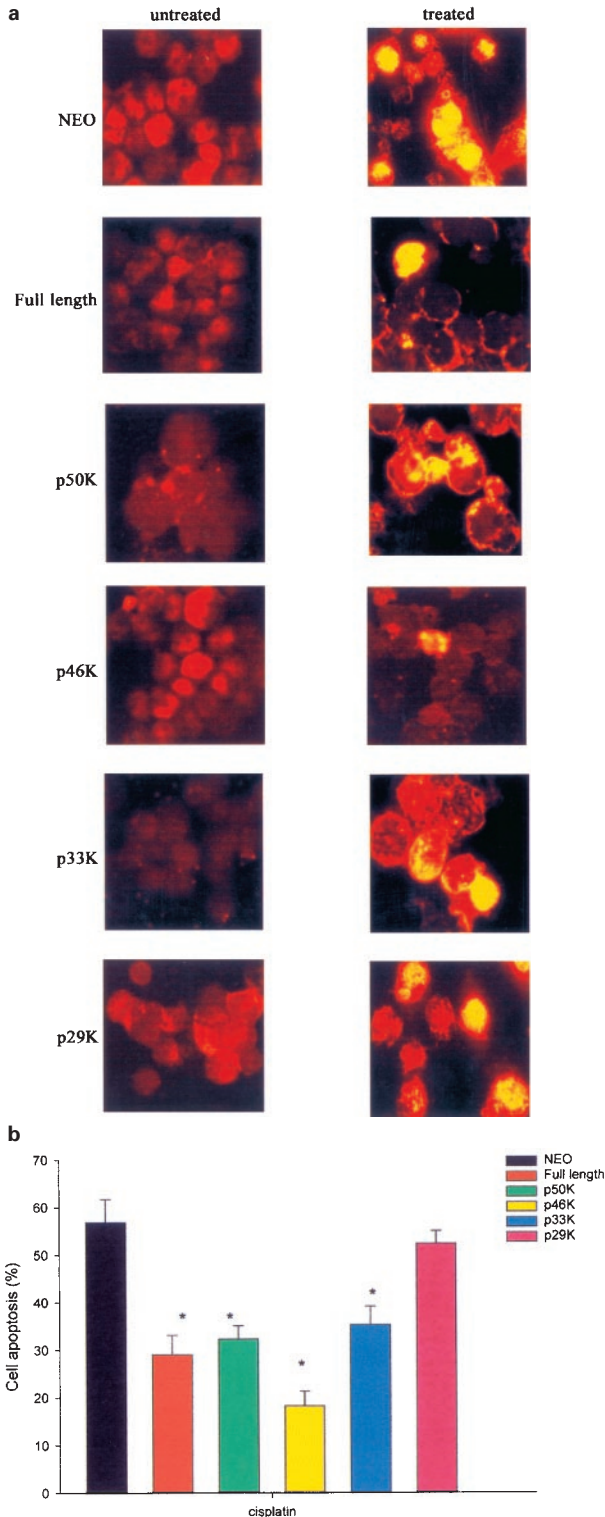


Figure 5 Effect of different isoforms of BAG-1 and cisplatin on apoptosis by TUNEL assay. 1×10^5 cells/well were seeded into 6-well plates, incubated for 24 h and treated with $10 \mu\text{M}$ cisplatin for 48 h. Apoptotic cells with characteristic nuclear fragmentation were counted in randomly chosen fields of at least 300 cells (a) and calculated as a percentage of the treated cells minus the percentage for untreated cells and presented as histogram (b). *Denotes the cell apoptosis that is statistically different from that of the cells transfected with NEO control ($P < 0.05$). The results represent four randomly chosen fields

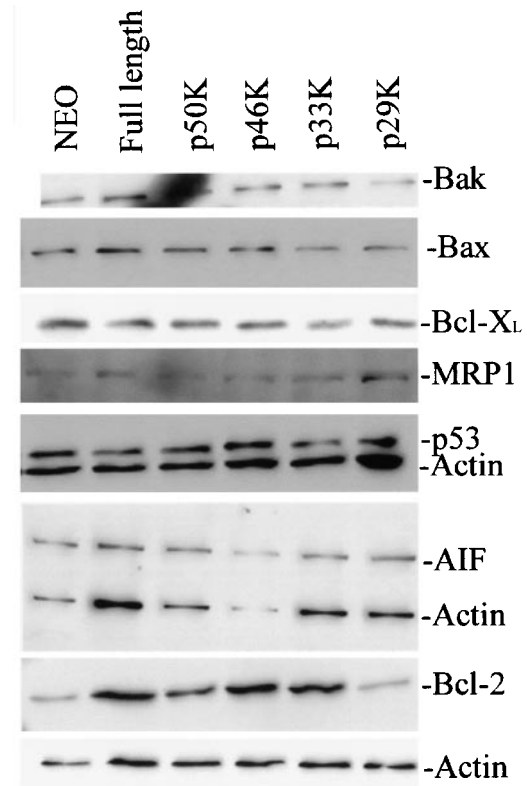


Figure 6 Expression of apoptosis and drug resistance-associated proteins in C33A cells stably transfected with the vector control plasmid pCR3.1 and plasmids containing BAG-1 full length and four isoforms cDNA. Western blot analysis is performed using $10 \mu\text{g}$ of protein from different cells. β -actin was used as an internal control. Expression of Bcl-2 was measured by densitometry

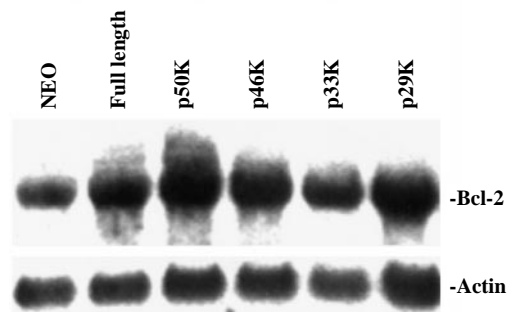


Figure 7 Expression of Bcl-2 RNA in cells transfected with different BAG-1 isoforms. For Northern blot, $20 \mu\text{g}$ of total RNA from cells were fractionated on a 1.0% agarose formaldehyde gel, transferred to nylon membranes, and hybridized with ^{32}P -dCTP-labeled Bcl-2 cDNA probe. Actin was used as an internal control

requires the presence of Bcl-2. BAG-1 may inhibit apoptosis through its interaction with other proteins such as protein kinases, heat shock proteins, hormone receptors or even directly with DNA promoters. There are four BAG-1 isoforms that are expressed differentially in different subcellular compartments and in different tissues (Yang *et al.*, 1998a; Brimmell *et al.*, 1999). Different BAG-1 isoforms have been reported to

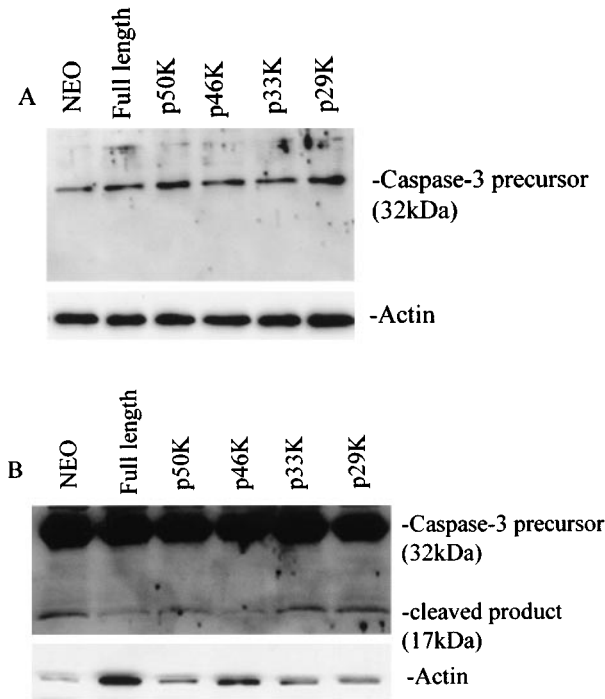


Figure 8 The effect of BAG-1 full length and distinct isoforms on caspase-3 activity and proteolytic activation in C33A cells treated with 10 μ M cisplatin for 48 h by Western blot analysis. (a) Untreated C33A cells stably transfected with NEO control and BAG-1 full length and different isoforms. (b) Caspase-3 activation induced by 10 μ M cisplatin, with the cleavage of the 32-kDa precursor into the 17-kDa activated caspase-3 protein

have different effects on heat shock protein function (Luders *et al.*, 2000b) and to possess different transcriptional activities (Zeiner *et al.*, 1999; Niyaz *et al.*, 2001). Isoform-specific BAG-1 expression during mouse embryonal development has also been noted (Crocoll *et al.*, 2000). It is likely that different BAG-1 isoforms perform different biological functions, such as anti-apoptosis.

Our study showed for the first time that forced expression of BAG-1 alone enables the BAG-1-transfected C33A cells to resist apoptosis induced by chemotherapeutic and chemical agents such as cisplatin, paclitaxel, doxorubicine, and staurosporine. In addition, different BAG-1 isoforms inhibited apoptosis differentially in response to different agents in our experiments. In C33A cells treated with all four agents, BAG-1 p46 exhibited the greatest degree of anti-apoptosis ($P < 0.05$), while BAG-1 p29 had little effect. BAG-1 p33 inhibited apoptosis in cells treated with cisplatin and staurosporine, but not in cells treated with paclitaxel or doxorubicine. Interestingly, C33A cells transfected with BAG-1 p50 demonstrated a similar resistance to apoptosis compared to those transfected with the native full-length BAG-1, even though the predominant BAG-1 isoform expressed was BAG-1 p33. Consistently, the differential ability of each BAG-1 isoform to inhibit apoptosis in response to different apoptotic agents was demonstrated by

Annexin-V FITC cell flow cytometry and by TUNEL assay. Finally, the decrease in apoptosis in the C33A cells transfected with the native full-length BAG-1, BAG-1 p50, and p46 was also demonstrated by the decreased activation of caspase-3, a down-stream molecule in the apoptosis signal transduction pathway. Our DNA fragmentation assays failed to detect any difference in apoptosis in the C33A cells transfected with each BAG-1 isoform and treated with different apoptotic agents. This is likely due to the lack of sensitivity of the assay in quantifying the degree of apoptosis in these cells.

The differential ability of BAG-1 in anti-apoptosis may be explained by the structural differences among the BAG-1 isoforms. All four BAG-1 isoforms share a common C-terminus which contains the BAG domain (Sondermann *et al.*, 2001) that interacts with Hsp 70 (Takayama *et al.*, 1997, 1999), Bcl-2 (Antoku *et al.*, 2001) and hepatocyte growth factor (HGF) receptor (Bardelli *et al.*, 1996). They also possess a common ubiquitin-like sequence that binds to proteasome and targets the molecular chaperones Hsc70 and Hsp70 to the protein degradation machinery (Luders *et al.*, 2000a). The ubiquitin-like domain is also important for BAG-1's anti-apoptotic function (Bardelli *et al.*, 1996; Luders *et al.*, 1998). BAG-1 p50 has the complete while BAG-1 p46 has the partial nuclear localization signal (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 (Yang *et al.*, 1998a). The exact mechanism by which BAG-1 participates in anti-apoptosis is currently unknown. The N-terminus seems to be important for BAG-1 to bind to hormone receptors (Froesch *et al.*, 1998; Kullmann *et al.*, 1998), transcription factors such as c-Fos (Zeiner *et al.*, 1997) and certain DNA promoters such as CMV promoter (Takahashi *et al.*, 2001). Our transfection experiments with BAG-1 isoforms showed that BAG-1 p46 had the strongest ability to suppress apoptosis, followed by BAG-1 p50 and p33, while BAG-1 p29 had no activity in anti-apoptosis. Our experiments are consistent with other publications which show that BAG-1 p46 is the most active isoform in the inhibition of hormone receptors (Kullmann *et al.*, 1998; Liu *et al.*, 1998 and Schneikert *et al.*, 1999) and in binding to DNA promoter (Takahashi *et al.*, 2001). The differential anti-apoptotic function of different BAG-1 isoforms suggests that the N-terminus of the protein is important for its function. Binding to Hsp70 and Bcl-2 through the BAG-1 domain and to proteasome via the ubiquitin-like region is obviously insufficient for BAG-1 to participate in anti-apoptosis. Furthermore, the resistance to apoptosis is likely mediated mainly by the cytosolic isoforms such as BAG-1 p46. Interestingly, BAG-1 p50 and p46 share a complete, while BAG-1 p33 shares a partial, hexapeptide repeat region in their N-terminus preceding the ubiquitin-like domain. BAG-1 p29 lacks this region (Figure 1, Luders *et al.*, 2000a). The function of this

hexapeptide repeat region is unclear. Deletion of this region renders the protein highly unstable (Luders *et al.*, 2000a). Search of PC-gene 6.85 data bank showed that this region contains many cAMP- and cGMP-dependent protein kinase and protein kinase C phosphorylation sites, suggesting that it may be involved in signal transduction. 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 has strong anti-apoptotic function. BAG-1 p33 with the incomplete hexapeptide repeat is anti-apoptotic only in response to cisplatin and staurosporine, but not to paclitaxel and doxorubicine, while BAG-1 p29 lacking the hexapeptide repeat does not have the ability to inhibit apoptosis. The presence of the hexapeptide repeat may also contribute to the suppression of the caspase-3 activation in the C33A cells transfected with the native full-length BAG-1, BAG-1 p50 and p46 in the apoptosis signal transduction pathway. BAG-1 isoforms with the incomplete or no hexapeptide repeats may be unable to suppress the caspase-3 activation. Finally, our experiment represents the first study to explore the function of BAG-1 p29, an isoform infrequently overexpressed in cancer cells. Although our study failed to show any activity of this isoform in anti-apoptosis in the C33A cells, it may still play some biological roles through its functional domains such as ubiquitin-like and BAG domain.

Our experiments showed for the first time that forced expression of BAG-1 alone is sufficient to cause increased resistance to apoptosis in C33A transfected with BAG-1 isoforms. Except for Bcl-2, transfection of BAG-1 does not change the expression of apoptotic proteins such as Bak, Bax, p53 and AIF and the multi-drug resistance protein MRP1. Bcl-2 protein expression was increased in cells transfected with the native BAG-1, BAG-1 p50, p46 and p33, but not with the p29 or the NEO, although the expression of Bcl-2 mRNA remained unchanged. Our experiments suggest that forced expression of certain BAG-1 isoforms may decrease by Bcl-2 protein degradation. BAG-1 has been reported to play an important role in protein folding and degradation through its interaction with the Hsp70 by the BAG domain (Takayama *et al.*, 1997) and with the proteasome by the ubiquitin-like region (Luders *et al.*, 2000b), respectively. All BAG-1 isoforms share a common C-terminus that binds to Hsp, Bcl-2 and proteasome. The increased expression of Bcl-2 in cells transfected with native BAG-1, BAG-1 p50, p46 and p33, but not with the BAG-1 p29 suggests that intact BAG-1 function is required for the inhibition of Bcl-2 protein degradation mediated by BAG-1. BAG-1 has been reported to cooperate with Bcl-2 to inhibit apoptosis (Takayama *et al.*, 1995). The increased resistance to apoptosis in the BAG-1-transfected cells cannot be simply attributed to the increased Bcl-2 expression, since cells transfected with BAG-1 p33 failed to resist the apoptosis induced by paclitaxel and doxorubicine, even though the expression of Bcl-2 was increased.

Our study demonstrates that different BAG-1 isoforms possess different abilities to suppress apoptosis in response to different apoptotic signals. Although forced expression of BAG-1 causes increased expression of Bcl-2, the anti-apoptotic function of BAG-1 seems to be largely determined by its N-terminal protein segment. The functional domains present on the N-terminus determine the subcellular distribution of the BAG-1 isoforms in the nucleus and cytosol. They may also participate in the transduction of apoptosis signals. BAG-1 subcellular distribution and isoform-specific expression therefore determines the BAG-1 function in specific tissues and cancer cells. Nuclear BAG-1 (p50 and p46) binds to hormone receptors and transcription factors and may function as a transcription or co-transcription factor to modulate expression of other apoptosis proteins. Cytoplasmic BAG-1 (p46, p33 and p29) binds to Hsp, protein kinases, Bcl-2 and/or growth factor receptors and modulate apoptosis differently.

Materials and methods

Construction of the recombinant plasmids carrying the native full-length and the four BAG-1 isoforms

Construction of plasmids containing the native full-length BAG-1, BAG-1 p50, p46, p33 and p29 were performed as described previously (Yang *et al.*, 1998a,b). Briefly, cDNA encoding different BAG-1 isoforms were amplified by PCR using different primer pairs (5' primers: p50K 5'-gccaccatggctcagcgcggggg-3'; p46K 5'-gccaccatggagaagaaaccgg-3'; p33K 5'-gccaccatggatcggagccagggt-3'; p29K 5'-gccaccatggcgcagctggctc-3' and 3' primer: 5'-tttctgtacacctactcgg-3') from approximately 100 ng of BAG-1 cDNA plasmid (Yang *et al.*, 1998a) and cloned into pCR3.1 plasmid vector (Invitrogen) in *EcoRI* site. For the native full-length BAG-1, hBAG-1 cDNA containing upstream CTG (nt 63) start codon (Yang *et al.*, 1998a) and the stop codon was cloned into pCR3.1 expression vector without the Kozak sequence (gccaccatgg) preceding the CTG start codon. For the BAG-1 p50, p46, p33 and p29 isoforms, cDNA containing upstream CTG (nt 63), first ATG (nt 276), second ATG (nt 422) and third ATG (nt 477) start codons (Yang *et al.*, 1998a) respectively, were PCR and cloned into pCR3.1 vectors with the Kozak sequence preceding each start codon. The PCR program was 94°C for 5 min for the first cycle; 94°C for 30 s, 60°C for 45 s, 72°C for 1 min for a total of 30 cycles; 72°C from 7 min for the last cycle; and 4°C on hold. All recombinant plasmids were confirmed by DNA sequencing.

Cell culture and transfection

C33A human cervical carcinoma cells that express very low level of BAG-1 protein were cultured in DMEM containing 10% fetal calf serum. DNA transfection was carried out using the Effectene Transfection Kit (Qiagen) according to the manufacturer's instruction. Briefly, 5×10^4 cells/well were seeded into 6-well plates and incubated at 37°C in 5% CO₂ overnight. Next day, 0.5 µg of plasmid DNA was mixed with Effectene Transfection Reagent and added onto the cell monolayer. 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 as cell lines for drug

treatment, apoptotic assays, mRNA and protein extraction, Northern and Western blot analysis.

Cell viability assay

5×10^3 cells/well were seeded into 96-well plates and incubated for 24 h. The cells were treated with $1 \mu\text{M}$ staurosporine, $10 \mu\text{M}$ cisplatin, $4 \mu\text{M}$ paclitaxel and $2 \mu\text{M}$ doxorubicine for 48 h. Cell viability was determined by Cell Titer 96[®] Aqueous One Solution Reagent Kit (Promega) according to the manufacturer's instruction. Cell viability was calculated as the percentage of the number of viable cells over the total number of cells.

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, then cells were treated with $1 \mu\text{M}$ staurosporine, $10 \mu\text{M}$ cisplatin, $4 \mu\text{M}$ paclitaxel and $2 \mu\text{M}$ doxorubicine for 48 h. Cells were stained using Annexin V-FITC Apoptosis Analysis Kit (PharMingen) and subjected to a FACStar plus flow cytometer (Becton-Dickenson) to sort out the Annexin V-FITC stained apoptotic cells. Data was analysed 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.

In situ cell apoptosis assay (TdT-mediated dUTP Nick End Labeling, TUNEL Assay)

1×10^5 cells/well were seeded into 6-well plates. After 24 h incubation, cells were treated with $10 \mu\text{M}$ cisplatin for 48 h, trypsinized from culture plates, fixed onto slides and stained using In Situ Cell Death Detection Kit (Roche). Apoptotic cells with characteristic nuclear fragmentation were counted (at least 300 cells) in four randomly chosen fields and calculated as the percentage of apoptotic cells in the treated cell population minus the percentage of apoptotic cells in the untreated control cell population.

DNA fragmentation analysis

Cellular DNA from drug-treated cells was extracted using Blood and Cell Culture Mini DNA Kit (Qiagen), analysed on

0.8% agarose gel and visualized by ethidium bromide staining under a UV transilluminator.

Northern blot analysis

Total RNA was extracted from 10^7 cells with RNeasy Mini kit (Qiagen). Northern blot analysis was performed as described (Yang *et al.*, 1997). In brief, $20 \mu\text{g}$ of total RNA was separated in denaturing 1% agarose gels and transferred to Hybond nylon membranes (Amersham). The blots were hybridized with ³²P-dCTP-labeled human Bcl-2 cDNA in Rapid-Hyb hybridization buffer (Amersham) at 65°C for 2 h, washed twice with $2 \times \text{SSC}$, 0.1% SDS at room temperature for 15 min and twice with $0.1 \times \text{SSC}$, 0.1% SDS at 65°C for 15 min, and then exposed to Kodak BioMax film at -70°C overnight. The Bcl-2 probe was stripped by using a boiling solution of 0.1% SDS and then re-hybridized with ³²P-dCTP-labeled human β -actin cDNA as internal control.

Western blot analysis

Cellular protein extraction and Western blot analysis were performed as described (Yang *et al.*, 1998a). Briefly, $10 \mu\text{g}$ of protein was fractionated on 10–12% SDS-PAGE and transferred onto Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membrane under semidry condition. Immunodetection was performed using ECL system (Amersham Pharmacia Biotech) (Figure 8). Monoclonal antibodies against Bax, Bak, p53, Bcl-2 and β -actin and polyclonal antibody against caspase-3, Bcl-X_L, AIF and MRP1 were purchased from Santa Cruz.

Statistical analysis

For cell flow cytometry, each experiment was duplicated and repeated three times. For cell viability assay, each experiment was triplicated and repeated at least three times. Data were analysed using Students' *t*-test. A $P \leq 0.05$ was considered statistically significant.

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