

# The anti-apoptotic protein BAG-3 is overexpressed in pancreatic cancer and induced by heat stress in pancreatic cancer cell lines

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**Abstract** Pancreatic cancer cells are usually resistant to apoptosis mediated by intrinsic or extrinsic factors. BAG-3 (Bis, CAIR), which was identified as a BAG-1-related protein, is a novel modulator of cellular anti-apoptotic activity that functions through its interaction with Bcl-2. In this study we analyzed BAG-3 expression in human pancreatic cancer tissues and cell lines. BAG-3 mRNA was expressed at moderate to high levels in all pancreatic cancer samples, but at low levels in normal pancreas tissues. In situ hybridization and immunohistochemistry analysis revealed that BAG-3 was present in the cancer cells within the pancreatic tumor mass. When BAG-3 mRNA was analyzed in other gastrointestinal cancers (hepatocellular carcinoma; esophageal, stomach and colon cancer), no difference was found from their corresponding normal controls. In pancreatic cancer cells, BAG-3 mRNA expression levels were strongly induced after heat stress, but not in response to members of the tumor necrosis factor (TNF)- $\alpha$  family (TNF- $\alpha$ , TRAIL, FasL). These findings indicate that in pancreatic cancer, in contrast to other gastrointestinal malignancies, increased levels of BAG-3 might function to block apoptosis. This characteristic of pancreatic cancer might contribute to its more aggressive growth behavior and poor responsiveness to treatment in vivo. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** BAG-3; Bis; CAIR; Pancreatic cancer; Gastrointestinal cancer; Apoptosis

## 1. Introduction

During the process of malignant transformation, pancreatic cancer cells acquire resistance to apoptosis through a number of mechanisms, including *p53* and *K-ras* mutations, aberrant expression of members of the Bcl-2-related gene family [1–3], and excessive activation of mitogenic pathways [4]. Pancreatic cancer cells are also resistant to apoptosis mediated by tumor necrosis factor (TNF)- $\alpha$  and FasL, and respond only poorly to TRAIL-mediated apoptosis [5–7]. This resistance, together with additional molecular alterations, contributes to unresponsiveness towards chemotherapy and/or radiotherapy in pancreatic cancer.

Intensive research has resulted in the identification of several proteins, which may promote carcinogenesis by inhibiting apoptosis. These proteins include, among others, members of the Bcl-2 family, which regulates apoptotic cell death and consists of several subfamilies, such as the anti-apoptotic Bcl-2 subfamily (Bcl-2, Bcl-xL and Bcl-W), the pro-apoptotic Bax subfamily (Bax, Bak and Bok), and the pro-apoptotic BH3 subfamily (Bad, Bik, Bid, Blk, Hrk, BNIP3 and BimL) [8,9].

Recent findings suggest that tumor cell death induced by chemotherapy and radiotherapy may be mediated by activation of apoptosis. The fact that pancreatic cancer has an extremely malignant potential and that it is resistant to most anti-cancer treatment modalities suggests that mechanisms are activated that increase the viability of pancreatic cancer cells. We have previously demonstrated enhanced expression of the anti-apoptotic gene Bcl-xL in pancreatic cancer and have shown that its enhanced expression is associated with shorter patient survival [2]. In line with this observation, high levels of Bcl-xL protect pancreatic cancer cells from FasL- and TRAIL-mediated apoptosis [10]. In addition, the anti-apoptotic gene Bcl-2 is overexpressed in approximately 1/3 of pancreatic cancer samples, yet there is no correlation between Bcl-2 expression and patient survival [1]. In contrast, the pro-apoptotic gene Bax was found to be overexpressed in approximately 2/3 of pancreatic cancers, and enhanced expression of this molecule correlated with longer survival of pancreatic cancer patients [1]. Other members of the Bcl-2 family have also been found to be aberrantly expressed in pancreatic cancer [3,11]. Collectively, these findings suggest that deregulation of the expression of members of the Bcl-2 family can enhance the viability of pancreatic cancer cells in vivo and that this might be one of the reasons why pancreatic cancers show only limited sensitivity to anti-cancer treatments.

BAG-3 (also known as Bis and CAIR) is a member of the Bag family of heat shock 70-kDa (Hsp70) chaperone regulators [12]. The BAG-3 protein contains a C-terminal Hsp70-binding 'BAG' domain, together with a WW domain. BAG-3 binds with high affinity to the ATPase domain of Hsc70/Hsp70, inhibiting its chaperone activity [12]. However, BAG-3 (Bis) has also been reported to associate with the anti-apoptotic protein Bcl-2 [13]. Functional analysis revealed that BAG-3 itself exerts only weak anti-apoptotic activity, but acts synergistically with Bcl-2 in preventing Bax-induced and FasL/Fas-mediated apoptosis [13]. The cellular functions are

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poorly understood. One report suggested that BAG-3 (CAIR) can associate with phospholipase-C following stimulation with epidermal growth factor (EGF) [12–14].

We have previously shown that another member of the BAG family, BAG-4 (SODD), is overexpressed in pancreatic cancer [15]. In view of the resistance of pancreatic cancer to apoptosis and the likely involvement of the BAG family in this process, in the present study we investigated the expression pattern of BAG-3 in diseased and normal human pancreatic tissues and analyzed the effects of heat shock and exposure to TNF- $\alpha$  family members on the expression of this molecule.

## 2. Materials and methods

### 2.1. Patients and tissue collection

Normal human tissue samples from the pancreas, liver, esophagus, stomach and colon were obtained through an organ-donor program from 20 individuals who were free of any apparent disease. The median age of the organ donors was 57 years, with a range of 27–73 years. Cancer tissue samples were obtained from 30 pancreatic cancer patients (11 female and 19 male, median age 65 years) undergoing pancreatic resection and from six liver, esophageal, stomach, and colon cancer patients undergoing tumor resection.

Freshly removed tissue samples were immediately fixed in paraformaldehyde solution for 12–24 h and paraffin-embedded for in situ hybridization and immunohistochemical staining. Concomitantly, tissue samples for RNA and protein extraction were immediately snap frozen in liquid nitrogen in the operating room upon surgical removal and maintained at  $-80^{\circ}\text{C}$  until use in Northern blot and Western blot analyses. The Human Subject Committee of the University of Bern approved all studies.

### 2.2. Cell culture

Human pancreatic cancer cells were routinely grown in Dulbecco's modified Eagle's medium (PANC-1, MIA-PaCa-2) or RPMI (T3M4, ASPC-1 and CAPAN-1) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 2 mM L-glutamine. Cells were maintained at  $37^{\circ}\text{C}$  in a humid chamber with 5%  $\text{CO}_2$  and 95% air atmosphere.

### 2.3. Cytokines and antibodies

Recombinant human TNF- $\alpha$  (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany), recombinant human TRAIL (Alexis Corp., Lausen, Switzerland) and agonistic monoclonal anti-Fas antibody (R&D Systems GmbH) were used for induction of apoptosis. The rabbit polyclonal anti-BAG-3 antibody was raised against a glutathione S-transferase (GST) fusion protein containing 196 amino acids of the C-terminal portion of BAG-3.

### 2.4. Probe synthesis for Northern blot analysis and in situ hybridization

A 201-bp fragment of human BAG-3 cDNA was amplified by RT-PCR using the following primers: forward: 5'-GAGAGACGGTGT-CAGGAAGG-3', reverse: 5'-GTGTGGGGATCTTCTGCATT-3'. The purified PCR products were subcloned into the pGEM-T Easy vector (Promega Biotechnology, Madison, WI, USA) according to the manufacturer's instructions. The identity of the cDNA fragments was confirmed by sequence analysis using the dye terminator method (Perkin Elmer, Rot Kreuz, Switzerland). A 190-bp fragment of mouse 7S that cross-hybridizes with human 7S was used to verify equivalent RNA loading and transfer in Northern blot analysis. For Northern blot analysis, the probes were radiolabeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP (Du Pont International, Regensdorf, Switzerland) using a random primer-labeling system (Roche Diagnostic Ltd., Rotkreuz, Switzerland). For in situ hybridization, digoxigenin-labeled BAG-3 cRNA sense and antisense probes were generated using the Ribomax System (Promega Biotechnology, Madison, WI, USA) and the appropriate polymerases, as described previously [16,17].

### 2.5. Northern blot analysis

The procedures used have been described in detail previously [16,17]. Briefly, following electrophoresis of total RNA in 1.2% aga-

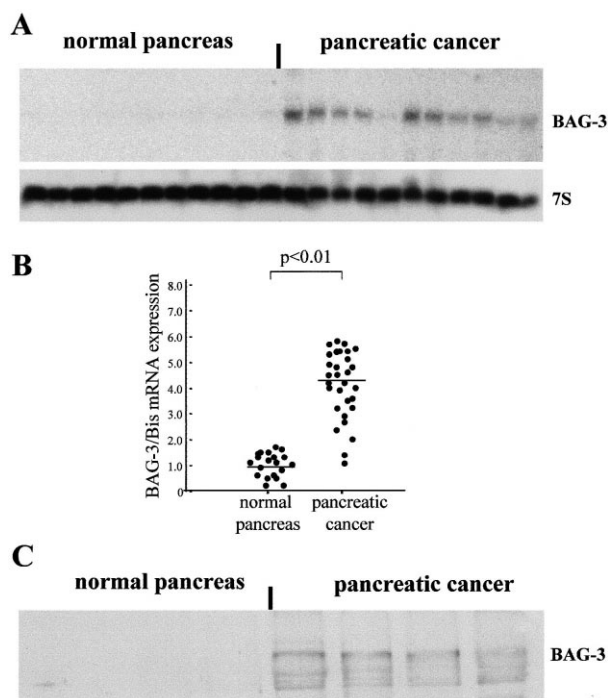


Fig. 1. Northern (A,B) and immunoblot (C) analysis of BAG-3 in pancreatic tissues. A: Total RNA (20  $\mu\text{g}$ ) isolated from normal and cancerous pancreatic tissues was subjected to Northern blot analysis and hybridized with the  $^{32}\text{P}$ -labeled BAG-3 cDNA probe. Blots were subsequently rehybridized with a 7S cDNA probe to verify equivalent RNA loading. B: Densitometry of the Northern blots. Relative BAG-3 mRNA expression was calculated as  $\text{OD}_{\text{BAG-3}}/\text{OD}_{7\text{S}}$  for each sample, and the fold increase over the mean in the normal pancreatic tissues was calculated. Horizontal lines represent the mean values of each group. C: Protein from each sample (40  $\mu\text{g}$ ) was subjected to immunoblot analysis using anti-BAG-3 antibodies, as described in Section 2. Antibody detection was performed with the enhanced chemoluminescence system.

rose/1.8 M formaldehyde gels, RNA was electrotransferred onto nylon membranes (Gene Screen, Du Pont, Boston, MA, USA) and cross-linked by UV irradiation. The filters were then pre-hybridized for 5 h at  $42^{\circ}\text{C}$  and hybridized for 20 h at  $42^{\circ}\text{C}$  in the presence of the radiolabeled cDNA probes for BAG-3 ( $10^6$  cpm/ml). Blots were then rinsed twice with  $2\times$  SSC at  $50^{\circ}\text{C}$  and washed twice with  $0.2\times$  SSC/2% sodium dodecyl sulfate (SDS) at  $55^{\circ}\text{C}$  for 20 min. All blots were exposed at  $-80^{\circ}\text{C}$  to Kodak XAR-5 film with Kodak intensifying screens, and the intensity of the radiographic bands was quantified by video image analysis using the Image-Pro plus software (Media Cybernetics, Silver Spring, MD, USA). To verify equivalent RNA loading on Northern blot membranes, filters were rehybridized with the 7S cDNA probe, as reported previously [16,17].

### 2.6. In situ hybridization

Tissue sections (4  $\mu\text{m}$ ) were deparaffinized, dehydrated, and incubated in 0.2 M HCl for 20 min. The sections were treated with proteinase K (50  $\mu\text{g}/\text{ml}$ ) for 15 min at  $37^{\circ}\text{C}$ . Following post-fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, the samples were pre-hybridized at  $60^{\circ}\text{C}$  for 2 h in 50% formamide (v/v),  $4\times$  SSC,  $2\times$  Denhardt's solution and 250  $\mu\text{g}/\text{ml}$  RNA. Hybridization was performed overnight at  $60^{\circ}\text{C}$  in 50% (v/v) formamide,  $4\times$  SSC,  $2\times$  Denhardt's solution, 500  $\mu\text{g}/\text{ml}$  RNA and 10% dextran sulfate (w/v). The final concentrations of the digoxigenin-labeled probes were approximately 0.5 ng/ $\mu\text{l}$ . After hybridization, the sections were washed and treated with RNase. The samples were then incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase (1:500). For color reaction, 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium (Sigma, Buchs, Switzerland) were used. For control experiments, the slides were incubated with RNase

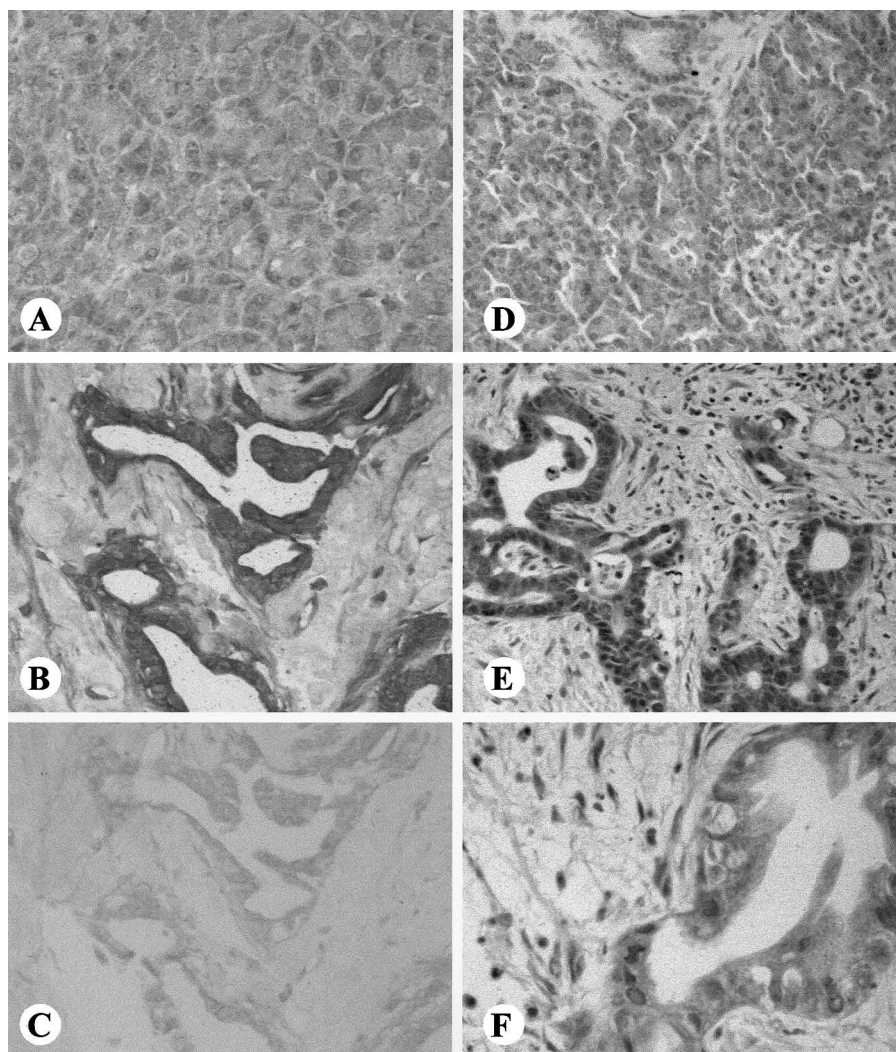


Fig. 2. Localization of BAG-3 mRNA and protein. In situ hybridization (A–C) was carried out with a BAG-3-specific digoxigenin-labeled anti-sense (A,B) and sense control (C) probe and anti-digoxigenin antibodies conjugated with alkaline phosphatase, as described in Section 2. Immunohistochemistry analysis (D–F) was performed with the streptavidin–peroxidase technique using anti-BAG-3 antibodies, as described in Section 2. Analyses were performed in the normal pancreas (A,D) and pancreatic cancer tissues (B–F).

or with the corresponding sense probes. Pre-treatment of the slides with RNase abolished the hybridization signals produced by the anti-sense probe. Furthermore, incubation with the sense probe failed to produce specific in situ hybridization signals.

### 2.7. Immunoblot analysis

Approximately 200 mg of frozen normal and cancer tissues were pulverized in liquid nitrogen and thawed in an ice-cold suspension buffer (10 mM Tris–HCl, pH 7.6, 100 mM NaCl) containing a proteinase inhibitor cocktail (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). Cultured pancreatic cancer cells ( $1 \times 10^6$ ) were washed in ice-cold PBS, scraped, centrifuged briefly, and lysed in 1 ml of ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% SDS), supplemented with the Compleat-TM mixture of proteinase inhibitors (Roche Diagnostics, Basel, Switzerland). Tissues and cells were homogenized for 5 min and then centrifuged (14000 rpm, 30 min at 4°C). The supernatants were collected and assessed for protein concentration with the micro BCA protein assay (Pierce Chemical Co., Rockford, IL, USA).

Protein from each sample (40 µg) was diluted in sample buffer (250 mM Tris–HCl, 4% SDS, 10% glycerol, 0.006% bromophenol blue and 2% β-mercaptoethanol), boiled for 5 min, cooled on ice for 5 min, and size-fractionated on 12% SDS–polyacrylamide gels. Gels were transferred onto nitrocellulose membranes at 100 V for 90 min.

Nitrocellulose membranes were incubated in a blocking solution

containing 5% non-fat milk in TBS-T (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20), followed by incubation with polyclonal rabbit anti-BAG-3 antiserum in blocking solution (0.1% v/v) for 1 h at room temperature. The membranes were then washed with TBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA, USA, 1:3000 dilution). Antibody detection was performed with the enhanced chemoluminescence immunoblot detection system (Amersham Life Science, Amersham, UK). The intensity of the bands was quantified by video image analysis using the Image-Pro plus software.

### 2.8. Immunohistochemistry

Immunohistochemical analysis was performed with the streptavidin–peroxidase technique, as previously reported [16,17]. Briefly, consecutive 3–5 µm paraffin-embedded tissue sections were deparaffinized and dehydrated. Incubating the slides in methanol containing 0.6% hydrogen peroxide blocked endogenous peroxidase activity. Then the sections were incubated for 30 min at room temperature with 10% normal goat serum prior to 1-h incubation at room temperature with the BAG-3 antibodies (3.4 ng/µl) diluted in 10% normal goat serum. Secondary biotinylated goat anti-rabbit IgG (Kirkegaard&Perry Laboratories Inc., Gaithersburg, MD, USA) were used. Slides were counterstained with Mayer's hematoxylin. To ensure specificity of the primary antibodies, consecutive tissue sections were incubated either in the absence of the primary antibody or with a non-

immunized rabbit IgG antibody. In these cases no immunostaining was detected.

### 2.9. Heat-stress experiments

After exposure to heat stress, BAG-3 mRNA and protein levels were analyzed by Northern blot analysis and immunoblot analysis. For Northern blot analysis, three pancreatic cancer cell lines (CAPAN-1, PANC-1 and T3M4) were heat-stressed at 45°C for 10, 30 or 60 min and mRNA was extracted following incubation at 37°C for 60 min. For immunoblot analysis, three pancreatic cancer cell lines (CAPAN-1, PANC-1 and T3M4) were heat-stressed at 45°C for 10 min and protein was extracted following incubation for 0, 1, 3, 6 or 12 h at 37°C.

### 2.10. BAG-3 induction experiments

CAPAN-1 cells ( $5 \times 10^4$ ) were cultured for 24 h in complete medium and subsequently treated with TNF- $\alpha$  (10 ng/ml), TRAIL+enhancer (100 ng/ml, 2  $\mu$ g/ml) or agonistic anti-Fas antibody (100 ng/ml) for 0, 1, 3, 6, 24 and 48 h at 37°C. Cells were harvested and analyzed by immunoblotting, as described above.

### 2.11. Statistical analysis

Results are expressed as median and range or mean  $\pm$  standard deviation (S.D.). For statistical analysis the Student's *t*-test was used. Values of  $P < 0.05$  were defined as significant.

## 3. Results

### 3.1. BAG-3 mRNA expression and protein levels in normal and cancerous pancreatic tissues

Normal pancreatic samples ( $n = 20$ ) and pancreatic cancer samples ( $n = 30$ ) were investigated by Northern blot analysis (Fig. 1A). The transcript size of BAG-3 mRNA was approximately 2.8 kb. The BAG-3 mRNA transcript was detected at moderate to high levels in all pancreatic cancer samples, but

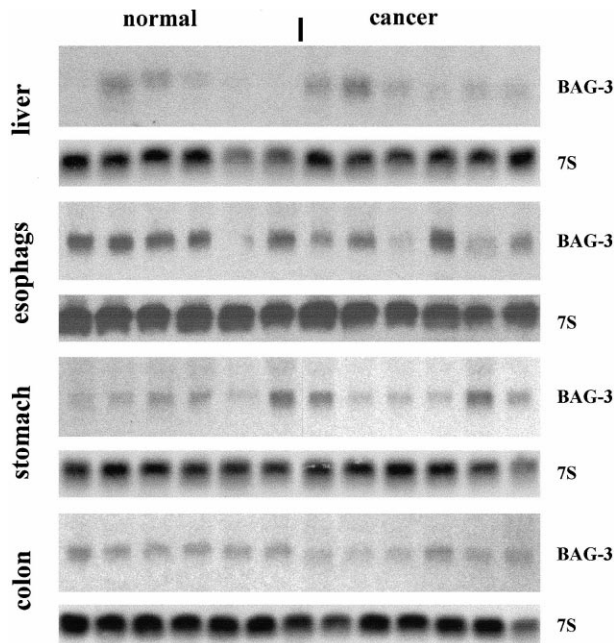


Fig. 3. Northern blot analysis of BAG-3 mRNA in liver, esophagus, stomach and colon tissues. Total RNA (20  $\mu$ g) isolated from the indicated normal and cancerous tissues was subjected to Northern blot analysis and probed with the  $^{32}$ P-labeled BAG-3 cDNA. Blots were subsequently rehybridized with the 7S cDNA probe to verify equivalent RNA loading.

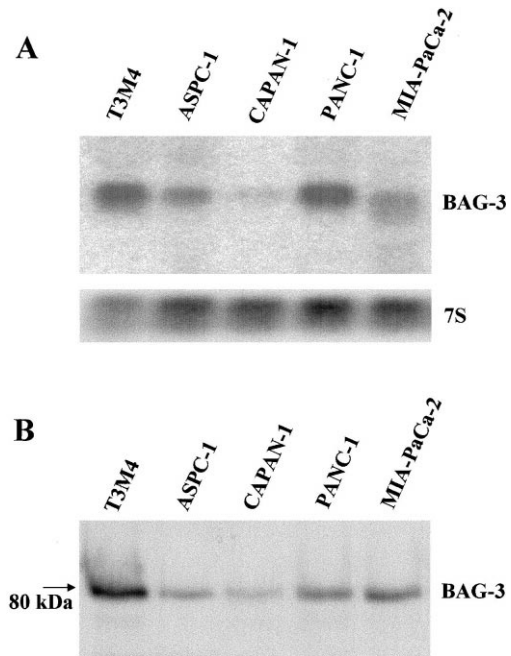


Fig. 4. Northern and immunoblot analysis of BAG-3 in pancreatic cancer cell lines. A: Total RNA (20  $\mu$ g) of five pancreatic cancer cell lines was subjected to Northern blot analysis and probed with the  $^{32}$ P-labeled BAG-3 cDNA. B: Protein samples (40  $\mu$ g) from five pancreatic cancer cell lines were subjected to immunoblot analysis using anti-BAG-3 antibodies, as described in Section 2. Antibody detection was performed with the enhanced chemoluminescence system.

at low levels in normal pancreatic tissue samples. Quantification of mRNA signals revealed that BAG-3 mRNA levels were  $4.2 \pm 0.9$ -fold increased ( $P < 0.01$ ) when all cancer samples were compared with the normal samples (Fig. 1B).

To determine whether BAG-3 protein levels were also elevated in pancreatic cancer, immunoblot analysis was carried out using four normal and four pancreatic cancer samples. The pancreatic cancers selected were those that exhibited the highest mRNA expression levels by Northern blotting. Immunoblot analysis revealed a single band of approximately 80 kDa, in accordance with the known size of BAG-3 [12]. The BAG-3 protein was present at higher levels in all pancreatic cancer samples compared to normal tissues (Fig. 1C). Densitometric analysis of the autoradiograms indicated that in comparison with normal pancreatic tissues, there was a  $5.4 \pm 0.8$ -fold increase ( $P < 0.01$ ) of BAG-3 protein levels in pancreatic cancer tissues.

### 3.2. Localization of BAG-3 mRNA and protein in the normal and cancerous pancreas

To assess the exact site of BAG-3 expression in human pancreatic cancer samples, in situ hybridization and immunohistochemistry were carried out next. BAG-3 mRNA signals were weak to absent in the normal pancreas (Fig. 2A). In contrast, BAG-3 mRNA signals were intense in the cytoplasm of cancer cells (Fig. 2B). Similarly, BAG-3 immunoreactivity was weak to absent in the normal pancreas (Fig. 2D). In contrast, in pancreatic cancer tissues, strong BAG-3 immunoreactivity was present in the cytoplasm of cancer cells within the pancreatic tumor mass (Fig. 2E,F).

### 3.3. BAG-3 expression in other normal and cancerous gastrointestinal organs

Tissue samples of other gastrointestinal cancers (hepatocellular carcinoma, esophageal cancer, stomach cancer and colon cancer) and the corresponding normal tissue samples were investigated by Northern blot analysis to determine whether differences in BAG-3 mRNA expression exist (Fig. 3). Interestingly, in contrast to the results observed in pancreatic cancer, BAG-3 mRNA levels in these gastrointestinal cancers were not obviously different from levels in the corresponding normal tissues (Fig. 3). Densitometric quantification of mRNA signals revealed that BAG-3 mRNA levels were not significantly changed ( $P > 0.05$ ) when cancer samples were compared with the respective normal samples.

### 3.4. BAG-3 expression in pancreatic cancer cell lines

Next, five human pancreatic cancer cell lines were investigated by Northern blot and immunoblot analysis. BAG-3 mRNA was detectable in T3M4, ASPC-1 and PANC-1 cells at high levels, and in CAPAN-1 and MIA-PaCa-2 cells at moderate levels (Fig. 4A). BAG-3 protein was strongly present in T3M4 and moderately present in the remaining pancreatic cancer cell lines (Fig. 4B).

### 3.5. Effect of heat stress on BAG-3 expression

We investigated whether heat stress induces an increase in BAG-3 levels. To this end, cells were heat-stressed at 45°C for

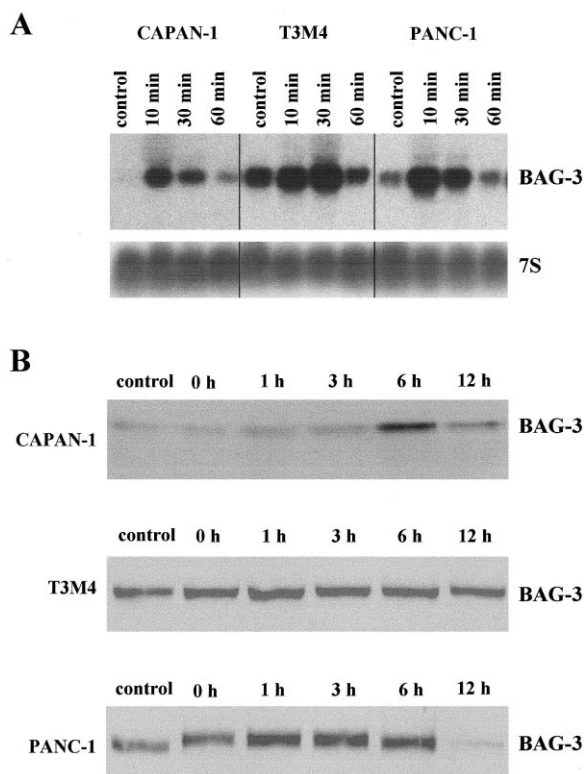


Fig. 5. Effect of heat stress on BAG-3 expression levels in pancreatic cancer cells. A: Cells were heat-stressed at 45°C for the indicated time and RNA was extracted following a 60-min incubation at 37°C. BAG-3 mRNA expression levels were determined by Northern blot analysis, as described in Section 2. B: Cells were heat-stressed at 45°C for 10 min and protein was extracted following incubation at 37°C for the indicated time. BAG-3 protein levels were determined by immunoblot analysis, as described in Section 2.

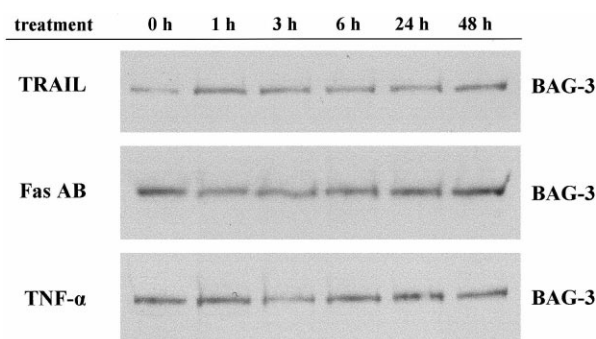


Fig. 6. Effect of TNF-family ligands on BAG-3 expression levels in CAPAN-1 pancreatic cancer cells. Cells were cultured for 24 h in complete medium and subsequently treated with 10 ng/ml TNF- $\alpha$ , 100 ng/ml TRAIL (and 2  $\mu$ g/ml enhancer), or 100 ng/ml agonistic anti-Fas antibody (Fas AB) for the indicated time. BAG-3 protein levels were determined by immunoblot analysis, as described in Section 2.

various times and RNA was extracted following a 60-min incubation period at 37°C. In CAPAN-1 and PANC-1 cells, maximal induction of BAG-3 mRNA occurred after 10 min of exposure, whereas longer heat stress of up to 60 min caused significantly less induction of BAG-3 mRNA expression. In contrast, in T3M4 cells, maximal induction of BAG-3 mRNA occurred after 30 min of exposure (Fig. 5A).

Based on the Northern blot findings, we investigated whether BAG-3 mRNA upregulation also leads to an increase in BAG-3 protein content. To this end, cells were heat-stressed at 45°C for 10 min and protein was extracted following incubation at 37°C for various times. The 10-min heat stress was chosen since all three cell lines exhibited a significant increase in BAG-3 mRNA expression at this time (Fig. 5A). In CAPAN-1 cells, BAG-3 protein levels increased slowly, with maximal levels observed 6 h after the 10-min heat stress. In PANC-1 cells, BAG-3 protein levels increased faster, yet maximal levels were also observed 6 h after the 10-min heat stress. In T3M4 cells, BAG-3 protein levels increased 1 h after the heat stress and decreased subsequently (Fig. 5B).

### 3.6. Effect of TNF-ligand treatment on BAG-3 expression

BAG-3 protein levels were analyzed by immunoblotting following treatment with TNF- $\alpha$ , TRAIL or agonistic anti-Fas antibodies. CAPAN-1 cells were utilized for these experiments since they displayed low-basal BAG-3 mRNA and protein levels with a strong induction following heat stress (Fig. 5). The levels of BAG-3 expression were not significantly changed following exposure to these three TNF ligands. Thus, there was no significant difference in BAG-3 levels at 0, 1, 3, 6, 24 and 48 h after treatment with the respective TNF ligands (Fig. 6).

## 4. Discussion

Apoptosis, or programmed cell death, is a normal physiologic process which occurs during embryonic development, as well as during maintenance of tissue homeostasis. In the course of cell division and differentiation, a surplus of cells is generated, which the normal organism eliminates by the activation of apoptotic signaling pathways [18]. Dereglulation

of apoptosis is commonly observed in cancer cells, and may play an important role in carcinogenesis [8].

Pancreatic cancer has one of the worst prognosis of all human malignancies, with an overall 5-year survival rate of less than 1% when all stages are combined [19,20]. One of the reasons for the dismal prognosis is the general resistance of pancreatic cancer cells to apoptosis induced extrinsically by either chemotherapy or radiotherapy [21–23]. Furthermore, pancreatic cancer cells are also resistant to intrinsic apoptosis mediated by members of the TNF- $\alpha$  family, such as FasL [5], TRAIL [6], and TNF- $\alpha$  [7]. The molecular mechanisms that enable pancreatic cancer cells to escape apoptosis are currently not well understood, although a number of possible mechanisms have been proposed, including deregulated expression of pro- and anti-apoptotic factors [1–3,11], excessive activation of mitogenic pathways, and mutations of tumor suppressor genes and oncogenes [4,24].

In the present study we analyzed the newly identified anti-apoptotic factor BAG-3 (Bis, CAIR) in pancreatic tissues. We now show that BAG-3 mRNA is expressed at moderate to strong levels in all pancreatic cancer samples, but is absent or only weakly present in the normal pancreas. In situ hybridization and immunohistochemistry analyses revealed that BAG-3 is present in the cancer cells within the pancreatic tumor mass. In contrast to the results in pancreatic cancer, BAG-3 mRNA levels in other gastrointestinal cancers were not obviously different from levels in the corresponding normal tissues. The observation of relatively high BAG-3 mRNA levels in some esophageal and gastric tissue specimens (normal, as well as cancerous) is interesting and warrants further investigation.

In addition, we show that in cultured pancreatic cancer cell lines, BAG-3 expression did not change following exposure to three TNF ligands, but was strongly induced following exposure to heat stress. Although heat-stress induction is artificial and usually does not occur in vivo, it elicits a variety of cytoprotective responses. Therefore, studying the effects of heat stress reveals the general capacity and the mechanisms of the exposed cells to avoid apoptosis.

The exact function of BAG-3 in pancreatic cancer is currently unknown. However, increased BAG-3 expression might contribute to a resistance to apoptosis via several mechanisms:

1. BAG-3 contains a conserved approximately 45-amino acid region, termed the BAG domain [12]. The BAG domain specifically binds and stimulates the ATPase activity of Hsp70/Hsc70 and modulates the function of these molecular chaperones. The homologous BAG-1 protein, which interacts with Hsp70/Hsc70, causes a wide variety of cellular effects, including increased resistance to apoptosis, enhanced cell proliferation, tumor cell migration and metastasis [12,25,26]. Interestingly, Hsp70 is commonly overexpressed in human tumors, and its expression in certain cancer types correlates with poor prognosis [27,28]. Hsp70 also protects tumor cells from TNF- $\alpha$  cytotoxicity even in the absence of heat treatment and various other stresses [28]. Therefore, it can be hypothesized that BAG-3 might also provide cytoprotective functions through its effects on Hsp70/Hsc70.
2. BAG-3 exhibits sequence similarity to SODD (BAG-4), which is a recently identified molecule that is reported to block spontaneous self association of TNF- $\alpha$  receptors, and thereby prevent apoptosis and NF $\kappa$ B induction [29]. While there is no experimental evidence that BAG-3 functions to block signaling of TNF- $\alpha$  members at the receptor level, it is intriguing to speculate that enhanced expression of BAG-3 could contribute to the observed resistance of pancreatic cancer cells to members of the TNF- $\alpha$  family, especially considering that there have been no convincing molecular explanations for this phenomenon. However, further research is required to explore this hypothesis.
3. BAG-3 may interact with Bcl-2, and thereby prevent apoptosis. There is experimental evidence that BAG-3 acts synergistically with Bcl-2 in preventing Bax-induced and Fas/FasL-mediated apoptosis [13]. In view of the fact that pancreatic cancers overexpress Bcl-2 in approximately 1/3 of the cases, and that these tumors almost uniformly overexpress BAG-3, it is possible that Bcl-2/BAG-3 interactions contribute to the apoptosis resistance in at least a subgroup of pancreatic cancer samples.
4. Recently it has been shown that BAG-3 (CAIR) forms an epidermal growth factor (EGF)-regulated complex with Hsp70/Hsc70 and latent phospholipase C- $\gamma$ , suggesting that BAG-3 may participate as a multifunctional signaling protein recruiting Hsp70/Hsc70 to pathways activated by the EGF receptor tyrosine kinase signaling complex [14]. In view of the excessive activation of mitogenic pathways of the EGF family in pancreatic cancer [4], it is possible that the increased BAG-3 levels in pancreatic cancers also contribute to the activation of mitogenic pathways in this disease, and thereby enhance the growth advantage of pancreatic cancer cells in vivo.

In conclusion, the precise function of BAG-3 in human malignancies is unknown, and it is currently not evident why enhanced BAG-3 mRNA levels are only observed in pancreatic cancer, but not in other gastrointestinal malignancies. While further studies are required to address these questions, the findings reported here provide preliminary evidence that BAG-3 plays a role in the pathogenesis or progression of pancreatic cancer.

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