Journal of Cellular Biochemistry 102:786–800 (2007)

A Novel Role for Bcl-2 Associated-Athanogene-1 (Bag-1) in Regulation of the Endoplasmic Reticulum Stress Response in Mammalian Chondrocytes

Ling Yang,[1] Denise McBurney,[1] Shou-Ching Tang,[2] Sara G. Carlson,[3] and Walter E. Horton, Jr.[1]*

[1]Department of Anatomy, Northeastern Ohio Universities College of Medicine, 4209 State Route 44, Rootstown, Ohio 44272
[2]Sylvester Comprehensive Cancer Center, 1475 N.W. 12th Avenue (D8-4), University of Miami, Miami, Florida 33136
[3]Stark State College of Technology, 6200 Frank Avenue NW, North Canton, Ohio 44720

Abstract BAG-1 (Bcl-2 associated athanogene-1) is a multifunctional protein, linking cell proliferation, cell death, protein folding, and cell stress. In vivo, BAG-1 is expressed in growth plate and articular cartilage, and the expression of BAG-1 is decreased with aging. Chondrocytes respond to endoplasmic reticulum (ER) stress with decreased expression of extracellular matrix proteins, and prolonged ER stress leads to chondrocyte apoptosis. Here we demonstrate for the first time that BAG-1 is involved in ER stress-induced apoptosis in chondrocytes. Induction of ER stress through multiple mechanisms all resulted in downregulation of BAG-1 expression. In addition, direct suppression of BAG-1 expression resulted in chondrocyte growth arrest and apoptosis, while stable overexpression of BAG-1 delayed the onset of ER stress-mediated apoptosis. In addition to regulating apoptosis, we also observed decreased expression of collagen type II in BAG-1 deficient chondrocytes. In contrast, overexpression of BAG-1 resulted in increased expression of collagen type II. Moreover, under ER stress conditions, the reduced expression of collagen type II was delayed in chondrocytes overexpressing BAG-1. These results suggest a novel role for BAG-1 in supporting viability and matrix expression of chondrocytes. J. Cell. Biochem. 102: 786–800, 2007.

Key words: BAG-1; Chondrocytes; ER stress

Bcl-2-asscociated athanogene (BAG) family proteins were originally identified by their ability to associate with Bcl-2 and enhance its antiapoptotic function [Takayama et al., 1995; Lee et al., 1999]. There are multiple BAG family members found in humans and animals [Doong et al., 2002] and BAG-1, the founding member of the family, is expressed as multiple isoforms generated by alternative translation initiation from a single mRNA [Yang et al., 1998]. All BAG-1 proteins have a 50 amino acid "BAG domain" near the C-terminus through which BAG-1 binds and activates the Hsp70 family of molecular chaperones [Takayama et al., 1997; Zeiner et al., 1999]. In addition, all BAG-1 isoforms contain an ubiquitin-like domain (ULD), which serves as an integral sorting signal to mediate the interaction between BAG-1 and the proteosome [Alberti et al., 2002]. Through the BAG domain and the ULD domain, BAG-1 proteins associate with a variety of cellular proteins and modulate specific pathways important for both normal and malignant cells, including signaling, proliferation, apoptosis, transcription, differentiation, embryogenesis, oncogenesis, and motility [Townsend et al., 2005]. Recent work suggests that BAG-1 may play a role in cell stress induced by hypoxia, radiation, and treatment with cytotoxic drugs [Townsend et al., 2003a]. In fact BAG-1 may play a role in moderating cell stress through multiple mechanisms including interaction with Raf-1 kinase and Hsp70 [Song et al., 2001], binding to Gadd34 [Hung et al., 2003],

*Correspondence to: Walter E. Horton, Jr., Northeastern Ohio Universities College of Medicine, 4209 State Route 44, Rootstown, OH 44240. E-mail: wehj@neocuom.edu

Received 18 January 2007; Accepted 12 February 2007
DOI 10.1002/jcb.21328

and regulation of protein folding and degradation [Agarraberes and Dice, 2001; Takamura et al., 2003].

A specific stress response in eukaryotic cells involves the endoplasmic reticulum (ER), a major storage organelle for calcium and site of synthesis and folding of secretory proteins, cell membrane proteins, and lysosomal proteins [Hammond and Helenius, 1995]. The ER is highly sensitive to alterations in calcium homeostasis and perturbations in its environment. Under physiological and pathological conditions, such as nutrient deprivation and oxidative stress, the function of the ER is impaired resulting in "ER stress" [Kaufman, 1999]. In order to overcome ER stress, cells respond by induction of unique genes such as the transcription inhibitor Gadd153 [Gotoh et al., 2004] along with the activation of pathways to inhibit protein accumulation in the ER as well as eliminate improperly folded proteins [Rao et al., 2004]. If the function of the ER is severely impaired, apoptosis may occur to eliminate the cells from the organism [Gotoh et al., 2004; Rao et al., 2004]. Although many aspects of the ER stress response are universal, certain features and consequences may be unique to different types of cells.

Cartilage is a specific connective tissue occupying about 10% of the total body tissue volume, and serves multiple functions in the developing embryo and in postnatal life [Kronenberg, 2003]. This tissue contains a single cell type, the chondrocyte, which is responsible for both synthesis and turnover of the abundant extracellular matrix (ECM). Chondrocytes experience a variety of stresses, such as osmotic stress, oxidative stress, and mechanical stress [Lee et al., 2002; Erickson et al., 2003; Loeser, 2004]. Also, existing within an avascular tissue, the chondrocyte is relatively sensitive to depletion of nutrients (such as glucose) and oxygen [Mobasheri et al., 2002]. Our previous work demonstrates that a variety of agents, including glucose deprivation, induce ER stress in chondrocytes, and that ER stress inhibits chondrocyte growth, causes downregulation of cartilage matrix expression, and ultimately induces chondrocyte apoptosis [Yang et al., 2005]. In addition, it has been reported that chondrocytes express BAG-1 and that the expression level declines with aging [Kinkel et al., 2004]. Further, suppression of BAG-1 expression results in decreased chondrocyte growth [Kinkel et al., 2004]. Also, Bcl-2 has been shown to be important in maintaining chondrocyte phenotype by inhibiting a MEK–ERK pathway that suppresses expression of genes coding for cartilage matrix [Yagi et al., 2005]. However, the relative roles of BAG-1 and Bcl-2 specifically related to ER stress have not been determined.

Here we present the novel finding that BAG-1 is a downstream mediator of the ER stress response. In addition, suppression of BAG-1 expression results in decreased ECM expression and the onset of apoptosis in chondrocytes. Conversely, overexpression of BAG-1 results in increased steady-state levels of collagen II under non-stress conditions as well as delays the onset of apoptosis and downregulation of collagen II in chondrocytes exposed to ER stressors.

MATERIALS AND METHODS

Cell Culture and Treatments

Immortalized rat chondrocytes (IRC) and primary rat articular chondrocytes (PC) were maintained in culture as previously described [Yang et al., 2005]. IRC cells with stable expression of BAG-1 were cultured in Ham's F-12 media in the presence of G418. Experiments were performed with cells seeded in monolayer culture at a density of 0.6×10^6 cells/well in 6-well plates. The cells were cultured in Ham's F-12 media (GibcoBRL, Grand Island, NY) +10% FBS, with or without glucose. The following treatments were used where indicated: 1 μg/ml Tunicamycin (Sigma, St. Louis, MO), 0.5 μM Thapsigargin (Sigma), 3 μg/ml retinoic acid (Sigma).

Stable Transfection

Linearized pRC/CMV/bag-1 (a generous gift from Dr. Devireddy, Memorial University of Newfoundland, Canada) was transfected into IRC by using the lipofectamine method as previously described [Kinkel et al., 2004]. The transfected cells were cultured in Ham's F-12 media with 10% FBS for 3–4 weeks in the presence of G418 to select the resistant cells. Several cell lines (including OE02 and OE15 used in this study) were selected based on the level of BAG-1 expression as detected by Western blot.

Adenoviral Vectors and Infection in Chondrocytes

Two RNAi oligoes were designed against rat bag-1. The oligonucleotide sequences are as follows: Oligo1: 5′-TCGA*GCAATGAGAGGTATGACCTTC*atcgat*GAAGGTCATACCTCTCATTGC*-3′; 3′-*CGTTACTCTCCATACTGGAAG*tagcta*CTTCCAGTATGGAGAGTAACG*GATC-5′; Oligo2: 5′-TCGA*TGGTCGTCACCCACAGCAAT*atcgat*ATTGCTGTGGGTGACGACCA*-3′; 3′-*ACCAGCAGTGGGTGTCGTTA*tagcta*TAACGACACCCACTGCTGGT*GATC-5′. The XhoI and XbaI overhang sites (underline) were added for ligation purpose; the loop region, which can be recognized by ClaI, is in lower case; rat Bag-1 specific sequences are in italics. The Bag-1 RNAi oligoes were ligated into pSuppressorAdeno (Imgenex, San Diego, CA), followed by cotransfected with pacAd5 plasmid into HEK 293A cells. The infected HEK 293A cells were collected and lysed. The cell lysates were used to infect HEK 293A cells for large scale viral amplification. After amplification, the viral particles were purified by the CsCl method. The virus titer was detected by using Adeno-X Rapid titer kit (BD Clontech, San Jose, CA). The AdenosiGFP plasmid (Imgenex) was used as a control RNAi, with the Adeno-x-lacZ (BD Clontech) used to detect the infection efficiency, as determined by β-galactosidase staining (Invitrogen, Carlsbad, CA). The chondrocytes were infected with Adneoviral RNAi using Lipofectamine2000 in Opti-MEM (Invitrogen) as previously described [Yagi et al., 2005].

Transient Transfection and Luciferase Assay

IRC cells cultured under 12-well plates under standard conditions as described above were transfected with Bag-1-luciferase construct (BGP-Luc, a generous gift from Dr. Tang, University of Miami) or control vector (pGL3-basic, Promega, Madison, WI) at 1.5 μg/well using LipofectAMINE 2000 (Invitrogen) according to the instructions of the manufacturer. The Bag-1-luciferase construct contains the Bag-1 5′ flanking sequence from −353 to −28, which displays promoter activity [Yang et al., 1999]. Transfected cells were cultured in complete medium for 24 h, then treated with glucose withdrawal, 1 μg/ml tunicamycin (Sigma) and 0.5 μM thapsigargin (Sigma) for 48 h, and collected for analysis. In specific cases, IRC cells were cotransfected with a Bag-1-luciferase construct (1.5 μg/well) and a full-length Gadd153 expression vector (pGFPCHOP, a generous gift from Dr. Maytin, Cleveland Clinic Foundation) or control vector (pEGFP-C1, Clontech, Palo Alto, CA) at 1.5 μg/well or 2.5 ug/well, and the transfected cells were cultured in complete medium for 48 h. Luciferase and β-galactosidase activity were detected with the Promega Reporter assay system (Promega). Luciferase activity was normalized against β-galactosidase activity. All values are expressed as mean ± standard deviation of three experiments carried out in duplicate.

Subcellular Fractionation

Cell fraction was done, as described [Zong et al., 2003]. Briefly, IRC chondrocytes (about 1×10^8 cells/ml) were resuspended in hypotonic buffer A (250 mM sucrose, 20 mMHepes, pH 7.5, 10 mM $MgCl_2$, 1 mMEDTA, 1 mM EGTA, 1× protease inhibitor) on ice for 30 min. Cells were disrupted by passing through 30-guage needles, and cell lysates were centrifuged at 750 g for 10 min at 4°C. The supernatant was centrifuged at 10,000*g* for 30 min at 4°C. The resulting pellet (mitochondrial fraction) was collected and the supernatant was further spun at 100,000*g* for 1 h at 4°C. The resulting pellet constituted the microsomal ER fraction and the supernatant constituted the cytosolic fraction. The mitochondrial and ER fraction were further lysed in the RIPA buffer.

Western Blot Analysis

IRC and primary rat chondrocytes were collected by centrifugation and resuspended in the RIPA buffer with protease inhibitors as previously described [Yang et al., 2005]. The membranes were blocked in 5% (W/V) nonfat milk 0.1% Tween TBS buffer for 2 h, and then incubated with the primary antibody overnight. Rabbit polyclonal anti-BAG-1 antibody (Santa Cruz, CA) was used at 1:1000 dilution; mouse monoclonal anti-Gadd153 (Santa Cruz) was used at 1:500 dilution; goat polyclonal anti-collagen II (Santa Cruz) was used at 1:1000 dilution; mouse monoclonal anti-Bcl-2 (BD Biosciences, San Jose) was used at 1:1000 dilution; mouse monoclonal anti-Bax clone 6A7 (Sigma,) was used at 1:2000 dilution; goat polyclonal anti-actin (Santa Cruz) was used at 1:6000 dilution. After four washes with 0.1% Tween TBS buffer, the membranes were

incubated with horse radish peroxidase (HRP)-linked anti-mouse, anti-rat, anti-rabbit or anti-goat secondary antibody at 1:10,000 dilutions. Following four washes with 0.1% Tween TBS buffer, immunoreactivity was visualized using an ECL system (Pierce, Rockford, IL).

Quantitative Real-time PCR

RNA isolation and reverse transcription were carried out as previously described [Yang et al., 2005]. Briefly, total RNA was isolated by using Trizol Reagent (Invitrogen). Genomic DNA was removed by treating with DNase (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) following the manufacturer instructions. Reverse transcription was performed at 37°C for 60 min. The quantitative PCR was performed as previously described [Yang et al., 2005]. Primers for quantitative real-time PCR were designed as previously described [Yang et al., 2005] and synthesized by Fisher Scientific. All primers were verified to have a similar efficiency of amplification.

Proliferation Assay and Apoptosis Assay

Proliferation assay was performed by using standard trypan blue staining. For DNA ladder assay, cytosolic DNA was prepared and analyzed on a 1.8 % agarose gel by electrophoresis as previously described [Yang et al., 2005]. Apoptosis was quantified using the APOPercentage Apoptosis Assay kit (Biocolor Ltd, Northern Ireland). Briefly, cells were cultured in 96-wells plate at 6×10^4 cells/well. After treatment with ER stress inducers, cells were stained with APOPercentage Dye for 60 min at 37°C, followed by washing with PBS. The positive cells/field were counted.

Immunocytochemistry

IRC cells were cultured in 4-well chamber slides at 0.2×10^6 cells/well for 24 h, followed by infection with Bag-1 RNAi or control RNAi. The Annexin V staining was performed according to the instructions of the manufacturer (Annexin V-FITC Apoptosis Detection Kit, CalbioChem, CA). Primary chondrocytes were cultured in 4-well chamber slides at 0.2×10^6 cells/well for 48 h, or cultured for 24 h followed by infection with Bag-1 RNAi or control RNAi. Cells were washed with PBS and fixed with 5% formalin for 20 min, followed by treatment with 0.2% Triton X-100 for 30 min at room temperature. The fixed and permeabilized cells were washed with PBS and blocked with 1% BSA for 1 hr at room temperature. Cells were incubated with an anti-Bag-1 antibody (Santa Cruz) for 2 h followed by a FITC-conjugated secondary antibody (Santa Cruz) at room temperature. The cell nucleus was stained with PI, and the stained cells were examined under a confocal laser microscope (Olympus, Fluoview FV300).

RESULTS

33 kDa BAG-1 is the Major Isoform in Chondrocytes

In a previous report, we showed that BAG-1 is expressed in chondrocytes in vivo [Kinkel et al., 2004]. To determine which specific BAG-1 isoform(s) are expressed in chondrocytes, a Western blot analysis was performed. According to manufacturer's instruction (Santa Cruz) and a previous report [Crocoll et al., 2000], the antibody used for detecting BAG-1 reacts with all major isoforms of human, mouse, and rat origin. As shown in Figure 1A, the major isoform of BAG-1 in two immortalized chondrocyte cell lines (IRC and RCS: rat chondrosarcoma), as well as in the primary rat chondrocytes is the 33 kDa BAG-1 isoform. We did not detect the longer isoform of BAG-1 in muscle of the postnatal rat, which is consistent with other

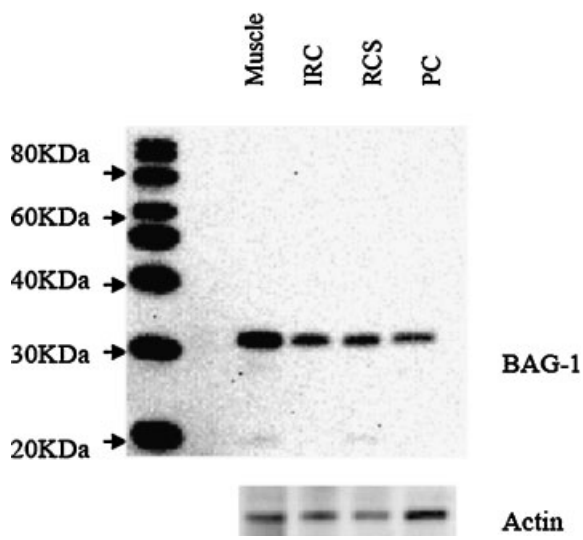

Fig. 1. The major isoform of BAG-1 in chondrocytes. BAG-1 protein expression in two immortalized chondrocyte cell lines, IRC (immortalized rat chondrocytes) and RCS (rat chondrosarcoma), the primary rat chondrocytes (PC) and the rat muscle cells (25 ug/well) were analyzed by Western blot analysis, with β-actin as a loading control.

studies showing that the BAG-1L isoform is only expressed up to certain stage of embryonic development [Crocoll et al., 2000]. Different isoforms and different subcellular distributions of BAG-1 have been suggested to have different functions [Chen et al., 2002; Townsend et al., 2005]. Therefore, we examined the localization of BAG-1 within the chondrocytes. Immunocytochemistry staining for BAG-1 in primary chondrocytes revealed that this isoform of BAG-1 is mainly distributed in the cytosol, especially in the perinuclear region (Fig. 3B).

BAG-1 is Downregulated in Chondrocytes Undergoing ER Stress

So far, no studies have demonstrated a role for BAG-1 in ER stress. However, given that BAG-1 can be an antiapoptotic protein [Tang, 2002; Townsend et al., 2005], and that prolonged ER stress results in chondrocytes undergoing apoptosis [Yang et al., 2005], we hypothesized that BAG-1 might play an important role in mediating the ER stress response in chondrocytes. To investigate this possibility, we examined whether the expression of BAG-1 would change during ER stress in chondrocytes. First, IRC were incubated with or without glucose for 24 h and 48 h, followed by measuring the BAG-1 expression level by Western blot assay. Withdrawal of glucose resulted in a three fold decrease in BAG-1 expression compared to the control at both time points (Fig. 2A). Over the same time course, the ER stress marker Gadd153 expression was induced with glucose

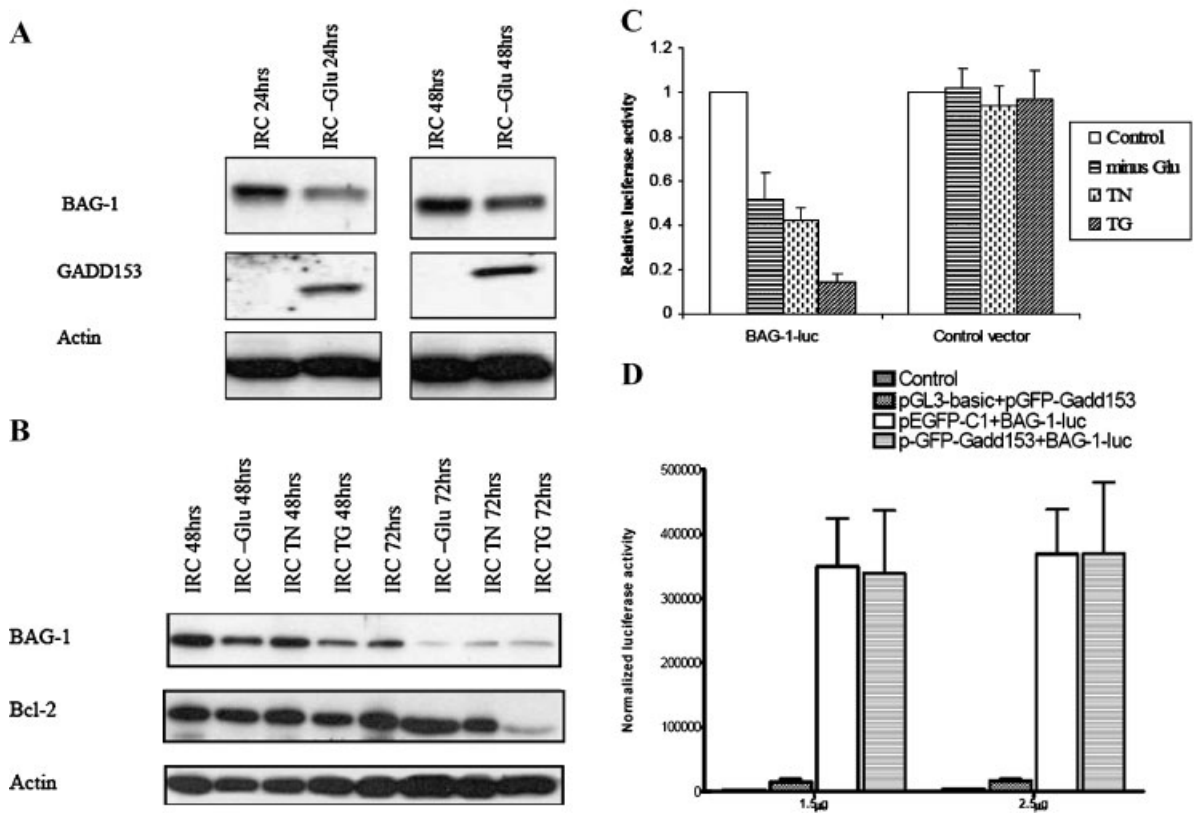

Fig. 2. BAG-1 is downregulated in chondrocytes exposed to ER stresses. (**A**) IRC cells were incubated in the presence and in the absence of glucose for 24 h and 48 h, followed by measuring the expression of BAG-1 by Western blot assay, using GADD153 as an ER stress marker. (**B**) IRC were cultured in the presence or absence of glucose (−Glu) or in the presence of tunicamycin (TN, 1μg/ml) and thapsigargin (TG, 0.5 μM) for 48 and 72 h. The BAG-1 and Bcl-2 expression levels were detected by Western blot analysis, with β-actin as a loading control. (**C**) IRC cells cultured in 12-well plates standard conditions were transfected with Bag-1-luciferase construct (BAG-1-luc) or control vector (pGL3-basic) at 1.5 μg/well using LipofectAMINE 2000. Transfected cells were cultured in complete medium for 24 h, and treated with glucose withdrawal, 1 μg/ml tunicamycin(TN) and 0.5 μM thapsigargin(TG) for 48 h, followed by collecting for analysis. Luciferase activity was normalized against β-galactosidase activity. The data are shown as the relative luciferase activity of treated cells compared to control which is set at 1.0. (**D**) IRC cells were cotransfected with Bag-1-luciferase construct (BAG-1-luc, 1.5 μg/well) with pEGFP-C1 or pGFP-Gadd153 at dose of 1.5 μg/well or 2.5 μg/well. The data are shown as the luciferase activity normalized against β-galactosidase activity. All values are expressed as mean ± standard deviation of three experiments carried out in duplicate.

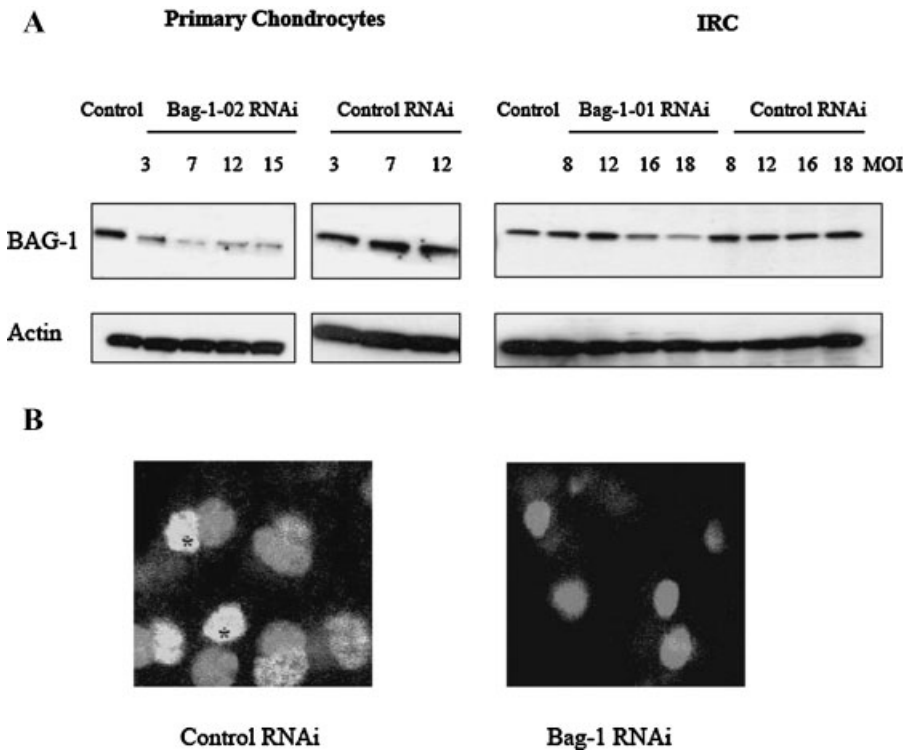

Fig. 3. The suppression of BAG-1 by RNAi. (**A**) Two adenoviral based small interference RNA (siRNA) were designed to target two regions each for rat Bag-1. IRC and primary chondrocytes were infected by adenoviral Bag-1-01 RNAi, Bag-1-02 RNAi, and control RNAi (GFP RNAi), followed by examining the protein level of BAG-1 by Western blot. MOI (multiplicity of infection). (**B**) Immunofluorescent staining for BAG-1 (labeled with asterisk) in primary chondrocytes, the nucleus was stained by PI. **Left**: control primary chondrocytes; **right**: primary chondrocytes infected with adenoviral Bag-1-02 RNAi.

withdrawal compared to control cells (Fig. 2A). In order to establish that the reduced BAG-1 expression was related to ER stress and not simply due to glucose withdrawal, we treated chondrocytes with two other additional ER stress inducers, tunicamycin, and thapsigargin. BAG-1 was downregulated by 48 h of exposure to all three ER stress inducers compared to control (Fig. 2B). BAG-1 may function in concert with Bcl-2 or work independently. Therefore, we next examined the expression of Bcl-2 in chondrocytes under ER stress conditions. No significant change in Bcl-2 expression was observed except for TG treatment at 72 h (Fig. 2B). Moreover, to test whether the down-regulation of BAG-1 protein in chondrocytes during ER stress is caused by downregulation of the transcriptional activity of BAG-1, we measured Bag-1 promoter activity. There was a decrease in Bag-1 promoter activity in chondrocytes exposed to all three ER stress inducers compared to control (Fig. 2C). Since we observed that Gadd153 is highly induced during chondrocyte ER stress, and Gadd153 is reported to be an important modulator of ER stress-mediated apoptosis through directly inhibiting Bcl-2 transcriptional activity [McCullough et al., 2001], we further asked whether Gadd153 could regulate BAG-1 promoter activity directly. As shown in Figure 2D, overexpression of Gadd153 did not affect the Bag-1 promoter activity.

A Direct Role for BAG-1 in Regulating Chondrocyte Growth and Apoptosis

We previously reported that chondrocytes with integrated antisense BAG-1 have a decreased growth profile in vitro [Kinkel et al., 2004]. In order to study the direct influence of BAG-1 on chondrocyte apoptosis, we generated adenoviral Bag-1 RNAi. IRC and primary chondrocytes were infected with adenoviral Bag-1-01 RNAi, Bag-1-02 RNAi, or control RNAi (Adenoviral GFP RNAi), followed by determining the protein level of BAG-1 by Western blot. As shown in Figure 3A, in primary

chondrocytes Bag-1-02 RNAi suppressed the expression of BAG-1 in a dose-dependent manner. A similar effect was observed with Bag-1-01 RNAi in IRC cells. In contrast, the control RNAi did not affect the expression level of BAG-1 in either cell types. Immunocytochemistry was used to confirm the altered expression of BAG-1 in chondrocytes. As shown in Figure 3B, Bag-1-02 RNAi suppressed cytoplasmic BAG-1 expression in the primary chondrocytes in situ compared to the control. Next, we determined the effect of directly suppressing BAG-1 on chondrocyte viability. We observed that knocking down BAG-1 with RNAi resulted in an inhibition of chondrocyte growth compared to cells infected with control RNAi (Fig. 4A). To establish whether the decreased growth kinetics caused by Bag-1 RNAi was due to the loss of viability of cells, we next proceeded to examine chondrocyte apoptosis. As shown in Figure 4B, suppression of BAG-1 by RNAi resulted in apoptosis of IRC chondrocytes as demonstrated by DNA ladder assay. In order to confirm these results, AnnexinV staining was performed on both IRC and primary chondrocytes infected with Bag-1 RNAi or control RNAi. Compared to the control there was a clear increase in AnnexinV staining in IRC with Bag-1 RNAi (Fig. 4C). Similar results were obtained with primary chondrocytes (data not shown).

Forced Expression of BAG-1 Delays the Onset of ER Stress-Induced Apoptosis

Since the antiapoptotic function of BAG-1 is reported to be associated with Bcl-2, we therefore examined the expression level of Bcl-2 in chondrocytes in the situation where Bag-1 expression is decreased as well as during overexpression of BAG-1. As shown in Figure 5A, there is no detectable change in the Bcl-2 protein level in either of these conditions.

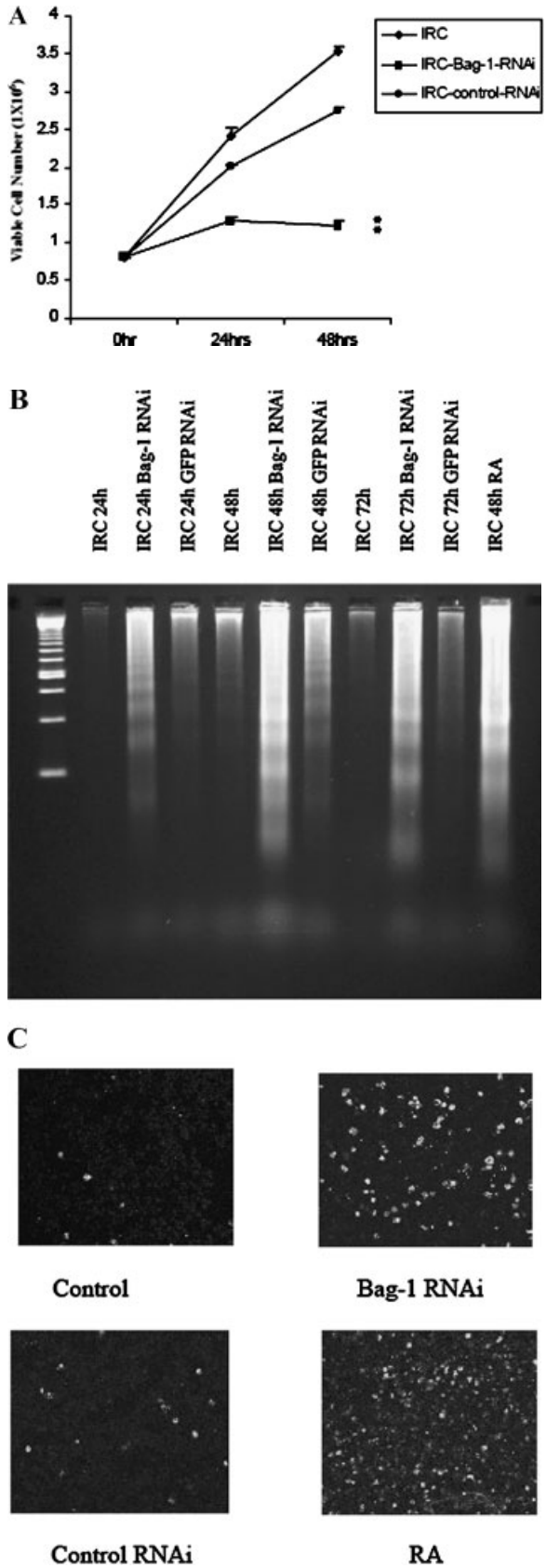

Fig. 4. Knocking down BAG-1 results in chondrocyte growth arrest and apoptosis. (**A**) IRC cells were infected with adenoviral Bag-1-01 RNAi (IRC-Bag-1-RNAi) or adenoviral GFP RNAi (IRC-control-RNAi) for 24 h and 48 h, and the viable cells were measured by trypan blue dye exclusion assay. (**B**) IRC cells were infected with adenoviral Bag-1RNAi or control RNAi. By 24 h, 48 h and 72 h, the DNA fragmentation was examined by DNA ladder, using retinoic acid (RA) treated IRC sample as positive control. (**C**) IRC cells were infected with adenoviral Bag-1RNAi and control RNAi for 48 h, followed by AnnexinV staining using retinoic acid (RA) treated IRC sample as positive control.

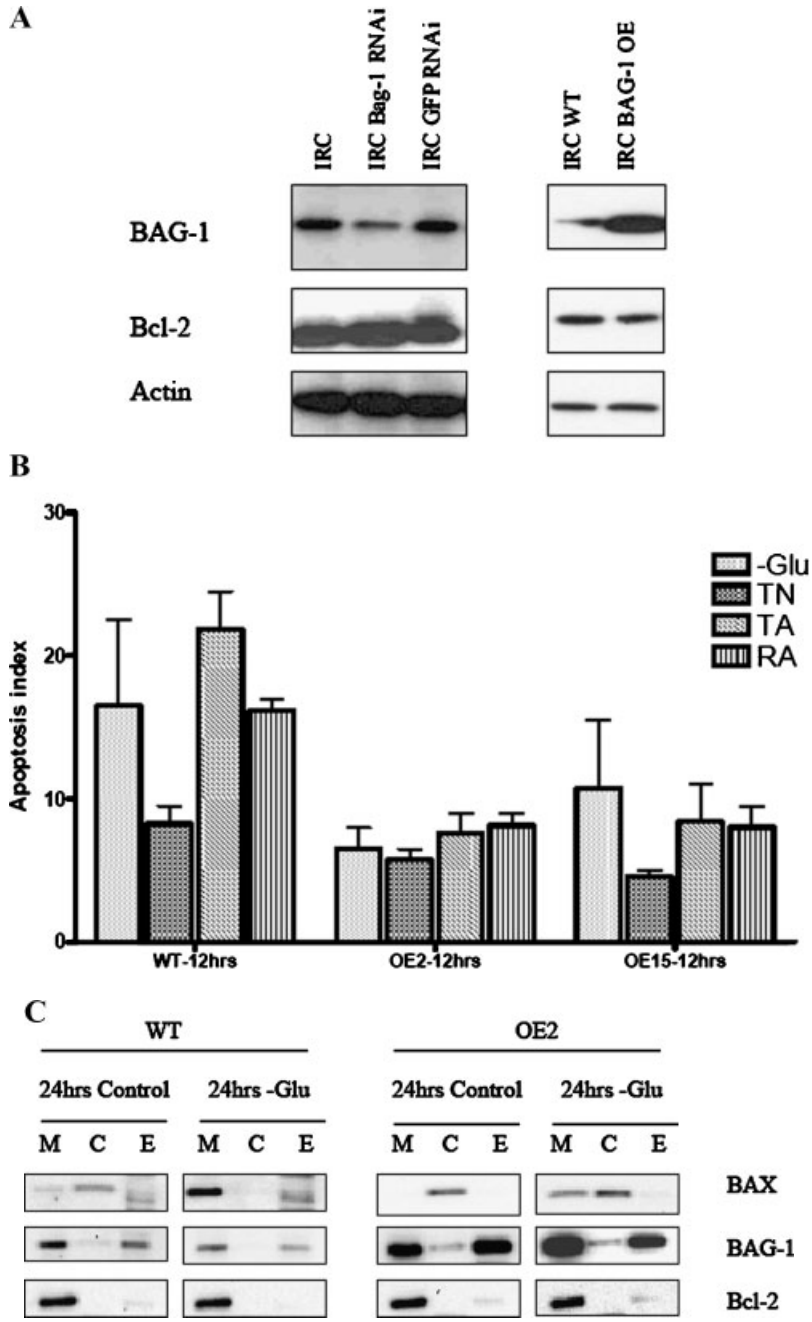

Fig. 5. Overexpression of BAG-1 delays the onset of ER stress-induced apoptosis. (**A**) IRC cells were infected with adenoviral Bag-1RNAi or control RNAi for 48 h, and cell lysates were collected and immunoblotted with anti-Bag-1 and anti-Bcl-2. Linerized pRC/CMV/bag-1 was transfected into IRC by using the Lipofectamine method. The IRC cells which stable express BAG-1 were collected after 48 h culture, and the expression levels of BAG-1 and Bcl-2 were detected by Western blot analysis. (**B**) Two BAG-1 overexpression IRC cell lines (OE2 and OE15) were treated with all of three ER inducers for 12 h, and the quantified apoptosis assay was performed by using an Apopercentage apoptosis assay kit. The apoptosis index is the ratio of number of apoptotic cells/field in treated cells to untreated cells in each cell line, with the control being set as 1.00. Data shown are the mean ± S.D of three independent experiments. (**C**) IRC wild-type (WT) and BAG-1 overexpression IRC cells (OE2) were treated with glucose withdrawal for 24 h. Cells were resuspended in hypotonic buffer A, and subjected to subcellular fractionation to obtain cytosolic (C), mitochondrial (M), and ER (E) fractions. 20 μg of protein/sample were separated on a 12% SDS–PAGE gel, and the BAX, BAG-1, and Bcl-2 expression levels were examined by immunoblotting.

To further establish the potential role of BAG-1 in ER stress-induced apoptosis in chondrocytes, a BAG-1 expression plasmid was introduced into IRC cells to generate chondrocyte cell lines with stable overexpression of BAG-1. After confirming the BAG-1 expression level by Western blot, wild type IRC and two Bag-1 IRC OE lines (OE-15 and OE-2) were treated with ER stressors. A time course study was carried out to detect the kinetics of apoptosis. Based on a previous study [Feng et al., 1998], we used RA treatment as a positive control for apoptosis. Apoptosis was quantified using an Apopercentage apoptosis assay kit. As shown in Figure 5B, apoptosis was evident at 12 h with all three treatments, which was consistent with our previous DNA ladder study. However, in two cell lines with stable overexpression of BAG-1 the level of apoptosis is significantly reduced (Fig. 5B). Extended exposure of chondrocytes to ER stress resulted in similar levels of apoptosis by 48 h in both control and BAG-1 OE cell lines (data not shown).

It is reported that the proapoptotic protein BAX is an essential component of the unfolding protein response (UPR) [Hetz et al., 2006], and that Gadd153 induces apoptosis associated with ER stress by mediating the translocation of BAX from the cytosol to the mitochondria, which is prevented by the Hsp70/dnaj pair [Gotoh et al., 2004]. It is also suggested [Townsend et al., 2003a] that the dynamic regulation of the BAG-1-Hsp70/Hsc70 interaction plays a critical role in controlling the cellular stress response. Therefore, we tested the hypothesis that BAG-1 regulates ER-stress-mediated apoptosis by preventing the upregulation and/or translocation of BAX to the mitochondria. Initial studies showed that the BAX level was increased in the cytosol of wild-type IRC chondrocytes 12 h after glucose withdrawal but not in the IRC expressing stable levels of BAG-1 (data not shown). More importantly, the majority of the BAX was translocated to the mitochondrial and ER membrane fractions in wild-type cells at 24 h after glucose withdrawal (Fig. 5C) which is a time point at which the steady-state level of BAG-1 is significantly reduced. However, the translocation of BAX was significantly blocked by stable expression of BAG-1 (Fig. 5C). In addition, we observed that BAG-1 is localized in both the mitochondrial and ER membrane fractions in wild-type IRC under non-stress conditions and the overall level of BAG-1 declines following glucose withdrawal (Fig. 5C). In contrast, the steady-state level of Bcl-2 does not change with glucose withdrawal. These data support a novel mechanism whereby BAG-1 attenuates ER stress-induced apoptosis by inhibiting the translocation of the pro-apoptotic molecule BAX.

BAG-1 is An Important Regulator of the Chondrocyte Phenotype

Besides its antiapoptotic function, it has been suggested that BAG-1 is important in chondrogenesis and in maintaining chondrocyte phenotype [Crocoll et al., 2002; Kinkel et al., 2004]. In this regard, we hypothesized that BAG-1 might play a role in regulating the expression of cartilage matrix proteins. To test this hypothesis, we examined the expression of type II collagen in BAG-1 overexpressing IRC cell lines OE2 and OE15, as compared to wild-type IRC. The cells were cultured in 6-well plates in complete medium with glucose to maintain homeostasis. After 36 h, cell lysates were prepared and analyzed by Western blotting. In addition, IRC cells were infected with Adeno-Bag-1 RNAi or control RNAi for 48 h, followed by Western blot assay. The BAG-1 overexpressing IRC cells have a 20-fold higher level of BAG-1 expression compared to control, while Bag-1 RNAi almost totally suppresses BAG-1 expression in IRC compared to cells infected with two control vectors (Fig. 6A). After confirming the expression level of BAG-1 in OE and adeno-Bag-1 RNAi cells, we next detected the collagen II expression level in these cells. As shown in Figure 6A, BAG-1 OE cells have a fivefold increase in the basal level of collagen II compared to wild-type IRC. By contrast, knocking down BAG-1 by RNAi almost completely abolished collagen II expression in cells, while the collagen II expression levels in both normal IRC and IRC infected with control RNAi were maintained. Interestingly, by 48 h of culture wild-type IRC cells showed increased endogenous BAG-1 expression compared to 36 h with an accompanying increased collagen II expression as well. The upregulated collagen II expression could be caused by increased transcription of collagen II, and/or increased stability of the protein. Thus, we further examined the expression level of mRNA coding for collagen II in wild type and BAG-1 overexpressing IRC cells. As shown in Figure 6B, there was about 2–3 increase in the mRNA level of collagen II in

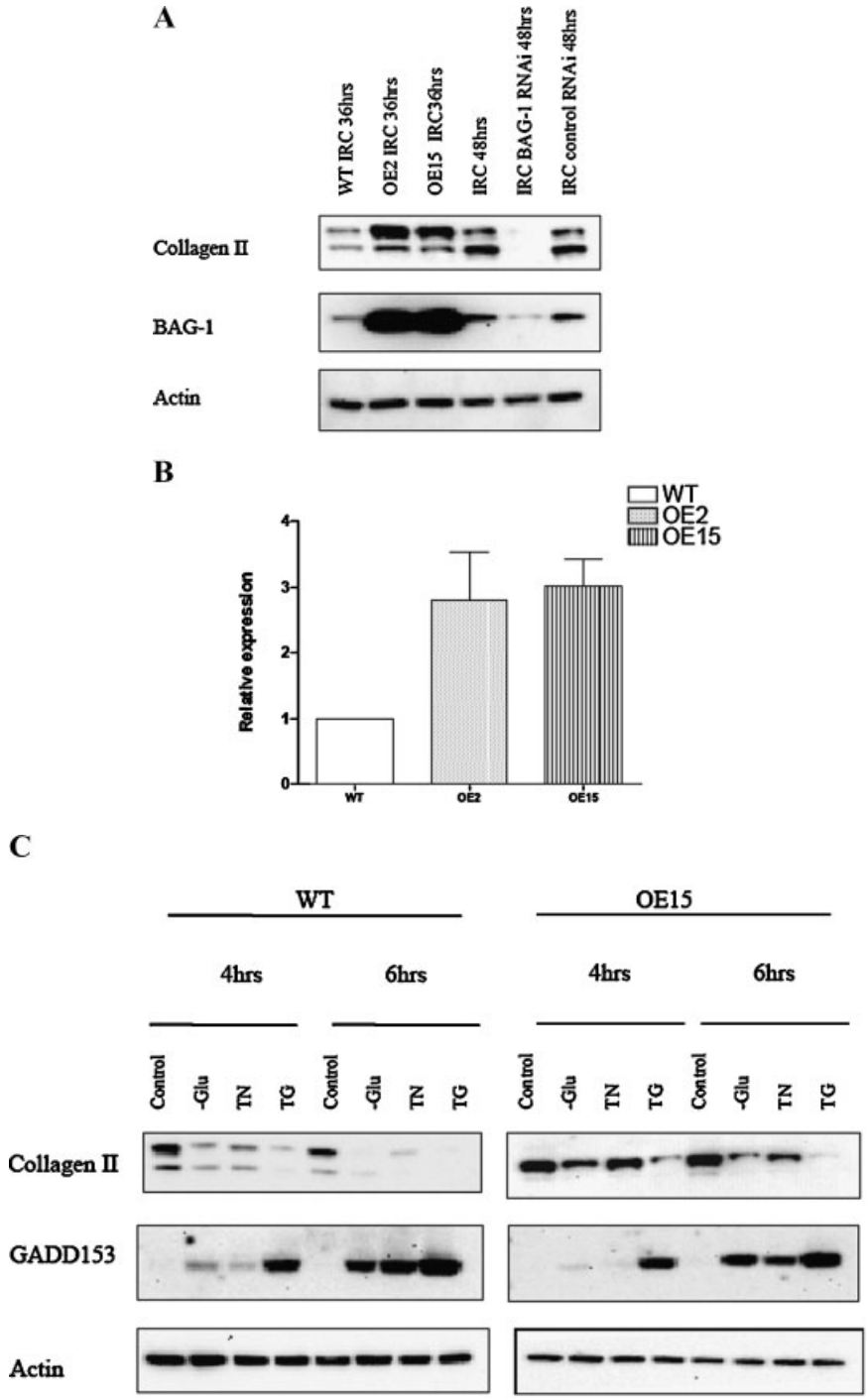

Fig. 6. BAG-1 overexpression delays the downregulation of collagen type II in chondrocytes during ER stress. (**A**) IRC cells were infected with adenoviral Bag-1RNAi and control RNAi (adenoviral GFP RNAi) for 48 h, as well as two BAG-1 overexpression IRC cell lines (OE-15 and OE-2) were culture for 36 h. The collagen II and BAG-1 expressions were examined by Western blot, using β-actin as a loading control. (**B**) The steady-state expression level of mRNA coding for type II collagen was determined by quantitative real time PCR in control (wild-type IRC, WT) and Bag-1 overexpressing IRC. The results are presented as relative expression of mRNA coding for type II collagen in BAG-1 overexpression of IRC cells versus wild-type IRC (WT). Data shown are the mean ± S.D. of three independent experiments. (**C**) Wild-type IRC (WT) and BAG-1 overexpressing IRC cell lines (OE15) were treated with glucose withdrawal, TN, and TG for 4 h and 6 h. Western blot analysis was performed to detect the expression levels of BAG-1 and GADD153, using β-actin as a loading control.

BAG-1 overexpressing cells compared to control. These data suggest that BAG-1 is critical for maintaining collagen II expression in chondrocytes.

BAG-1 Overexpression Delays the Downregulation of Collagen Type II Expression During Chondrocyte ER Stress

Previously, we reported that during chondrocyte ER stress there was decreased expression of collagen II [Yang et al., 2005]. Here we show that chondrocyte ER stress is accompanied by decreased expression of BAG-1. We next asked whether or not BAG-1 could function to maintain collagen II expression during ER stress. Wild-type IRC and a BAG-1 overexpressing IRC cell line (OE-15) were treated with glucose withdrawal, TN, or TG for 4 h and 6 h. As shown in Figure 6C, although both types of cells show some loss of collagen II expression by 4 h, BAG-1 OE cells maintained the expression of collagen II at higher levels for a longer time period than wild-type IRC following treatments with all three ER stress inducers. However, with all three treatments both wild-type and OE IRC cells lose collagen II expression at 12 h (data not shown), at which time point the cells start to undergo apoptosis. These data suggest that when chondrocytes initially develop ER stress, BAG-1 can protect cells from losing collagen II expression. However, if cells are exposed to ER stress for longer time periods, BAG-1 cannot prevent the loss of phenotype and viability. In addition, the expression of Gadd153 was induced by 4 h with TG treatment, and by 6 h with TN and glucose withdrawal treatments in both wild-type and BAG-1 expressing IRC cells. These results indicate that increased BAG-1 did not inhibit the expression of Gadd153 during ER stress, suggesting that Gadd153 was not located downstream of BAG-1 in the chondrocyte ER stress pathway.

DISCUSSION

BAG-1 is a multifunctional protein working through binding and modulating the function of a variety of cellular proteins [Townsend et al., 2005]. Here we report for the first time that BAG-1 is involved in ER stress-induced apoptosis. Further, we show that BAG-1 plays an important role in chondrocyte viability and phenotype regulation. Specifically, knocking down the expression of BAG-1 results in chondrocyte cell growth arrest and apoptosis. Conversely, stable overexpression of BAG-1 delays the onset of ER stress-induced apoptosis. We also observed decreased expression of collagen type II in BAG-1 deficient chondrocytes, while overexpression of BAG-1 resulted in increased expression of collagen II. Moreover, under ER stress conditions, the inhibition of collagen type II expression was delayed in chondrocytes with stable BAG-1 expression.

Previously, we demonstrated [Kinkel et al., 2004] that BAG-1 is expressed in mice growth plate and articular cartilage in vivo, and that the expression level declines with age in mice. Here we show that in vitro the major isoform of BAG-1 in rat chondrocytes (both in primary and immortalized chondrocytes) is the 33 kDa non-nuclear BAG-1 form. This result is consistent with the observation of another group, demonstrating that in vivo the 33 kDa BAG-1 is the most abundant BAG-1 isoform found in adult rat epithelium [Takamura et al., 2003]. Additionally, the immunocytochemistry results show that this BAG-1 isoform is predominantly localized in the perinuclear region in primary chondrocytes. Furthermore, the cell fractionation results demonstrate that BAG-1 is mainly localized to mitochondria and ER, indicating the potential role of BAG-1 in regulating organelle homeostasis and organelle-mediated apoptosis in chondrocytes.

The chondrocyte is a particularly useful cell type for studying the role of BAG-1 in ER stress. Chondrocytes are the only resident cell in the cartilage, and are critical for maintaining the normal function of cartilage. Cartilage, as an avascular tissue, is exposed to a variety of stressors, and ER stress has been linked to impaired chondrocyte function [Oliver et al., 2005; Yang et al., 2005] and cartilage diseases [Hecht et al., 1995; Luo et al., 1996; Hecht and Sage, 2006]. In the current study, we demonstrate for the first time that BAG-1 is downregulated at both the transcriptional and protein level in chondrocytes during ER stress induced by both physiological signals and pharmacological agents. To detect whether ER stress in chondrocytes causes a general loss of antiapoptotic proteins, we examined the expression of Bcl-2, which is reported to play an important role in ER stress-mediated apoptosis in certain cells [Breckenridge et al., 2003]. Interestingly, Bcl-2 expression was mainly affected only in the TG treated cells, while

BAG-1 was downregulated with all three ER stressors. Thapsigargin is a selective inhibitor of the Ca^{+}-ATPase (SERCA) of the ER, which potentially induces ER stress by disturbing ER calcium [Berridge et al., 2000]. ER stress results in the release of calcium from the ER and the subsequent transport of calcium to the mitochondria, which triggers the release of cytochrome C, and induces downstream apoptosis [Rao et al., 2004]. Based on our observations, we proposed that BAG-1 might act as a general ER stress modulator and plays an important role in the cellular response to multiple stress stimuli. Moreover, we previously demonstrated [Yang et al., 2005] that apoptosis starts to occur at 12 hrs following induction of ER stress in chondrocytes. Here, we observe that Bcl-2 levels are maintained for up to 72 h in TG treated cells, while the downregulation of BAG-1 starts earlier (Fig. 2B). These results imply that downregulation of BAG-1 is an earlier event than loss of Bcl-2 in chondrocytes, and that losing the expression of BAG-1 may sensitize cells to stress-induced apoptosis.

Previous studies have suggested that BAG-1 can limit apoptosis induced by a variety of signals [Schulz et al., 1997; Chen et al., 2002; Townsend et al., 2003b]. In the present study, we further demonstrate that knocking down BAG-1 by RNAi results in chondrocyte apoptosis. However, the induced apoptosis is not due to a deficiency of Bcl-2, since there is little change of expression of Bcl-2 relative to BAG-1 (Fig. 2A). Additionally, the expression of Bcl-2 is also not affected by overexpression of BAG-1. These results indicate that the antiapoptotic function of BAG-1 might be to some extent independent of Bcl-2 in chondrocytes. Our results differ from other studies that have shown that the expressions of BAG-1 and Bcl-2 are coupled. For example, decreased Bcl-2 expression has been reported in Hela cells following treatment with antisense BAG-1 [Xiong et al., 2003]; also increased Bcl-2 following overexpression of BAG-1 has been reported in C33 cells [Chen et al., 2002]. This difference might be due to the different expression patterns and functions of BAG-1 in different systems.

Townsend et al. [2003a] hypothesized that stress-induced cell death pathways are the primary targets of BAG-1 pathways involved in preventing apoptosis. In this regard, we further examined the effect of BAG-1 on the chondrocyte ER stress response. We show that overexpression of BAG-1 in chondrocytes results in delaying ER stress-induced apoptosis as compared to wild-type chondrocytes, although apoptosis did eventually occur in the BAG-1 overexpressing cells. These observations are consistent with the suggestion of Townsend that the effects of BAG-1 overexpression are rather modest and reflect a delay in the kinetics rather than full prevention of cell death.

Gadd153 (Growth arrest and DNA damage-inducible gene; also referred to as CHOP, C/EBP homologus protein) is a member of the C/EBP family of transcription factors, which is either not expressed or expressed at low levels under physiological conditions, and is highly induced by ER stress [Gotoh et al., 2004]. It was reported that Gadd153 regulated ER stress-induced apoptosis by directly inhibiting the Bcl-2 expression at the transcriptional level [McCullough et al., 2001]. It is possible that BAG-1 might be either upstream or downstream of Gadd153. Thus, to establish how BAG-1 functions in ER stress-induced apoptosis, we detected the expression level of Gadd153 in both wild-type and BAG-1 overexpressing cells (Fig. 6C). We observed that the Gadd153 expression level was similar in wild-type and BAG-1 overexpressing chondrocytes subjected to ER stress, indicating that BAG-1 is not upstream in a pathway that inhibits expression of Gadd153 in the ER stress signal pathway. In addition, the transcriptional activity of BAG-1 is not changed in chondrocytes overexpressing Gadd153, indicating Gadd153 is not the direct upstream regulator of BAG-1 during chondrocyte ER stress. Based on these observations, we hypothesize that BAG-1 is regulated through a different pathway as compared with Bcl-2 during ER stress in chondrocytes. In fact, it is reported that BAG-1 can regulate other Bcl-2 family members such as BAD by associating with Raf-1 and Akt [Gotz et al., 2005]. Moreover, it has also been shown that Gadd34, another Gadd family protein, is highly induced during ER stress [Kojima et al., 2003] and in vitro, the human BAG-1 protein interacts with GADD34, therefore masking GADD34-mediated growth arrest and suppression of transcription under cell stress conditions [Hung et al., 2003].

Given the diversity of targets and cellular effect, BAG-1 can be regarded as a candidate protein with important functions in cartilage. Of note, it is demonstrated that the stage and

site specific expression of BAG-1 has an important role in mouse embryonic development [Crocoll et al., 2000, 2002]. Our previous study [Kinkel et al., 2004] suggested that BAG-1 might be an important regulator of the chondrocyte phenotype. In this regard, we next examined the effect of BAG-1 on the differentiated phenotype of chondrocytes. Here we show evidence that, besides protecting cell viability, BAG-1 overexpression in IRC promotes the chondrocyte phenotype. First, collagen II expression is almost completely abolished following knock down of BAG-1 expression. Conversely, the expression level (at both mRNA and protein level) of collagen II is increased in chondrocytes overexpressing BAG-1. However, the upregulation of collagen II may not solely be caused by increased transcription, since the increased fold of mRNA levels of collagen II is less than the increased protein level in BAG-1 overexpressing chondrocytes compared to wild-type cells (Fig. 6B). Therefore, we propose that BAG-1 might play an important role in regulating collagen II expression through both transcriptional regulation and possibly stabilizing the processing of procollagen in the ER. In fact, a general function of BAG-1 in the protein secretion pathway has been reported in the literature. For example, Atsushi et al. reported that BAG-1 is targeted to the COPI-coated structure adjacent to the Golgi stack, implying its contribution in COPI vesicular transport in gastrointestine epithelium cells [Takamura et al., 2003].

The ER is the cytoplasmic compartment where extracellular proteins are synthesized and modified. Cells respond to ER stress by reducing new synthesis of protein, and accelerating ER stress associated unfolded protein degradation [Kaufman, 1999]. We previously reported [Yang et al., 2005] that there was significantly reduced cartilage matrix expression at both the mRNA and protein level during chondrocyte ER stress, and the downregulated ECM expression occurred before the onset of apoptosis. In addition to regulating cell proliferation and cell survival, the BAG-1 family proteins are also thought to play an important role in facilitating or controlling protein refolding and turnover by coordinating the activity of the chaperone and proteasome systems [Luders et al., 2000; Alberti et al., 2003]. Thus, we further hypothesize that BAG-1 might act as a modulator regulating chondrocyte matrix protein expression under stress conditions. In this study, we demonstrate that BAG-1 not only plays an important role in maintaining chondrocyte phenotype under normal conditions, but also during ER stress conditions, since in the absence of ER stress-induced apoptosis, the collagen II expression was maintained for a certain period in BAG-1 overexpressing cells exposed to ER stressors compared to wild type cells. In the future, we will focus on examining how BAG-1 regulates the expression of collagen II under ER stress conditions as well as during homeostasis.

In summary, our results suggest that BAG-1 plays an important role in maintaining chondrocyte survival and differentiated phenotype. In addition, the current study presents a novel role for BAG-1 in modulating the ER stress-induced cell apoptosis that may be applicable to many cell types.

ACKNOWLEDGMENTS

We thank Dr. Edward V. Maytain, of Cleveland Clinic Foundation, for kindly providing the pEGFPCHOP construct, and Amy Hilger for assistance with cloning the RNAi constructs.

REFERENCES

Agarraberes FA, Dice JF. 2001. A molecular chaperone complex at the lysosomal membrane is required for protein translocation. J Cell Sci 114:2491–2499.

Alberti S, Demand J, Esser C, Emmerich N, Schild H, Hohfeld J. 2002. Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. J Biol Chem 277:45920–45927.

Alberti S, Esser C, Hohfeld J. 2003. BAG-1–a nucleotide exchange factor of Hsc70 with multiple cellular functions. Cell Stress Chaperones 8:225–231.

Berridge MJ, Lipp P, Bootman MD. 2000. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1:11–21.

Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. 2003. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 22:8608–8618.

Chen J, Xiong J, Liu H, Chernenko G, Tang SC. 2002. Distinct BAG-1 isoforms have different anti-apoptotic functions in BAG-1-transfected C33A human cervical carcinoma cell line. Oncogene 21:7050–7059.

Crocoll A, Blum M, Cato AC. 2000. Isoform-specific expression of BAG-1 in mouse development. Mech Dev 91:355–359.

Crocoll A, Herzer U, Ghyselinck NB, Chambon P, Cato AC. 2002. Interdigital apoptosis and downregulation of BAG-1 expression in mouse autopods. Mech Dev 111: 149–152.

Doong H, Vrailas A, Kohn EC. 2002. What's in the 'BAG'?—A functional domain analysis of the BAG-family proteins. Cancer Lett 188:25–32.

Erickson GR, Northrup DL, Guilak F. 2003. Hypo-osmotic stress induces calcium-dependent actin reorganization in articular chondrocytes. Osteoarthritis Cartilage 11:187–197.

Feng L, Precht P, Balakir R, Horton WE Jr. 1998. Evidence of a direct role for Bcl-2 in the regulation of articular chondrocyte apoptosis under the conditions of serum withdrawal and retinoic acid treatment. J Cell Biochem 71:302–309.

Gotoh T, Terada K, Oyadomari S, Mori M. 2004. hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. Cell Death Differ 11:390–402.

Gotz R, Wiese S, Takayama S, Camarero GC, Rossoll W, Schweizer U, Troppmair J, Jablonka S, Holtmann B, Reed JC, Rapp UR, Sendtner M. 2005. Bag1 is essential for differentiation and survival of hematopoietic and neuronal cells. Nat Neurosci 8:1169–1178.

Hammond C, Helenius A. 1995. Quality control in the secretory pathway. Curr Opin Cell Biol 7:523–529.

Hecht JT, Sage EH. 2006. Retention of the matricellular protein SPARC in the endoplasmic reticulum of chondrocytes from patients with pseudoachondroplasia. J Histochem Cytochem 54:269–274.

Hecht JT, Nelson LD, Crowder E, Wang Y, Elder FF, Harrison WR, Francomano CA, Prange CK, Lennon GG, Deere M, et al. 1995. Mutations in exon 17B of cartilage oligomeric matrix protein (COMP) cause pseudoachondroplasia. Nat Genet 10:325–329.

Hetz C, Bernasconi P, Fisher J, Lee AH, Bassik MC, Antonsson B, Brandt GS, Iwakoshi NN, Schinzel A, Glimcher LH, Korsmeyer SJ. 2006. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. Science 312:572–576.

Hung WJ, Roberson RS, Taft J, Wu DY. 2003. Human BAG-1 proteins bind to the cellular stress response protein GADD34 and interfere with GADD34 functions. Mol Cell Biol 23:3477–3486.

Kaufman RJ. 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13:1211–1233.

Kinkel MD, Yagi R, McBurney D, Nugent A, Horton WE Jr. 2004. Age-related expression patterns of Bag-1 and Bcl-2 in growth plate and articular chondrocytes. Anat Rec 279A:720–728.

Kojima E, Takeuchi A, Haneda M, Yagi A, Hasegawa T, Yamaki K, Takeda K, Akira S, Shimokata K, Isobe K. 2003. The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress: Elucidation by GADD34-deficient mice. Faseb J 17:1573–1575.

Kronenberg HM. 2003. Developmental regulation of the growth plate. Nature 423:332–336.

Lee JH, Takahashi T, Yasuhara N, Inazawa J, Kamada S, Tsujimoto Y. 1999. Bis, a Bcl-2-binding protein that synergizes with Bcl-2 in preventing cell death. Oncogene 18:6183–6190.

Lee RB, Wilkins RJ, Razaq S, Urban JP. 2002. The effect of mechanical stress on cartilage energy metabolism. Biorheology 39:133–143.

Loeser RF Jr. 2004. Aging cartilage and osteoarthritis—what's the link? Sci Aging Knowledge Environ 2004: pe 31.

Luders J, Demand J, Papp O, Hohfeld J. 2000. Distinct isoforms of the cofactor BAG-1 differentially affect Hsc70 chaperone function. J Biol Chem 275:14817–14823.

Luo W, Kuwada TS, Chandrasekaran L, Zheng J, Tanzer ML. 1996. Divergent secretory behavior of the opposite ends of aggrecan. J Biol Chem 271:16447–16450.

McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21: 1249–1259.

Mobasheri A, Vannucci SJ, Bondy CA, Carter SD, Innes JF, Arteaga MF, Trujillo E, Ferraz I, Shakibaei M, Martin-Vasallo P. 2002. Glucose transport and metabolism in chondrocytes: a key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis. Histol Histopathol 17:1239–1267.

Oliver BL, Cronin CG, Zhang-Benoit Y, Goldring MB, Tanzer ML. 2005. Divergent stress responses to IL-1beta, nitric oxide, and tunicamycin by chondrocytes. J Cell Physiol 204:45–50.

Rao RV, Ellerby HM, Bredesen DE. 2004. Coupling endoplasmic reticulum stress to the cell death program. Cell Death Differ 11:372–380.

Schulz JB, Bremen D, Reed JC, Lommatzsch J, Takayama S, Wullner U, Loschmann PA, Klockgether T, Weller M. 1997. Cooperative interception of neuronal apoptosis by BCL-2 and BAG-1 expression: Prevention of caspase activation and reduced production of reactive oxygen species. J Neurochem 69:2075–2086.

Song J, Takeda M, Morimoto RI. 2001. Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. Nat Cell Biol 3:276–282.

Takamura A, Adachi M, Wada I, Mitaka T, Takayama S, Imai K. 2003. Accumulation of Hsp70/Hsc70 molecular chaperone regulator BAG-1 on COPI-coated structures in gastric epithelial cells. Int J Oncol 23:1301–1308.

Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. 1995. Cloning and functional analysis of BAG-1: A novel Bcl-2-binding protein with anti-cell death activity. Cell 80:279–284.

Takayama S, Bimston DN, Matsuzawa S, Freeman BC, Aime-Sempe C, Xie Z, Morimoto RI, Reed JC. 1997. BAG-1 modulates the chaperone activity of Hsp70/Hsc70. Embo J 16:4887–4896.

Tang SC. 2002. BAG-1, an anti-apoptotic tumour marker. IUBMB Life 53:99–105.

Townsend PA, Cutress RI, Sharp A, Brimmell M, Packham G. 2003a. BAG-1 prevents stress-induced long-term growth inhibition in breast cancer cells via a chaperone-dependent pathway. Cancer Res 63:4150–4157.

Townsend PA, Cutress RI, Sharp A, Brimmell M, Packham G. 2003b. BAG-1: A multifunctional regulator of cell growth and survival. Biochim Biophys Acta 1603:83–98.

Townsend PA, Stephanou A, Packham G, Latchman DS. 2005. BAG-1: A multi-functional pro-survival molecule. Int J Biochem Cell Biol 37:251–259.

Xiong J, Chen J, Chernenko G, Beck J, Liu H, Pater A, Tang SC. 2003. Antisense BAG-1 sensitizes HeLa cells to

apoptosis by multiple pathways. Biochem Biophys Res Commun 312:585–591.

Yagi R, McBurney D, Horton WE Jr. 2005. Bcl-2 positively regulates Sox9-dependent chondrocyte gene expression by suppressing the MEK-ERK1/2 signaling pathway. J Biol Chem 280:30517–30525.

Yang X, Chernenko G, Hao Y, Ding Z, Pater MM, Pater A, Tang SC. 1998. Human BAG-1/RAP46 protein is generated as four isoforms by alternative translation initiation and overexpressed in cancer cells. Oncogene 17:981–989.

Yang X, Pater A, Tang SC. 1999. Cloning and characterization of the human BAG-1 gene promoter: upregulation by tumor-derived p53 mutants. Oncogene 18:4546–4553.

Yang L, Carlson SG, McBurney D, Horton WE Jr. 2005. Multiple signals induce endoplasmic reticulum stress in both primary and immortalized chondrocytes resulting in loss of differentiation, impaired cell growth, and apoptosis. J Biol Chem 280:31156–31165.

Zeiner M, Niyaz Y, Gehring U. 1999. The hsp70-associating protein Hap46 binds to DNA and stimulates transcription. Proc Natl Acad Sci USA 96:10194–10199.

Zong WX, Li C, Hatzivassiliou G, Lindsten T, Yu QC, Yuan J, Thompson CB. 2003. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 162:59–69.