BAG3 Regulates Motility and Adhesion of Epithelial Cancer Cells

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Abstract

BAG3 protein binds to and regulates Hsp70 chaperone activity. The BAG3 protein contains a WW domain and a proline-rich region with SH3-binding motifs, suggesting that it may interact with proteins relevant to signal transduction, recruiting Hsp70 to signaling complexes and altering cell responses. BAG3 overexpression has been observed in human cancers. We show here that homozygous BAG3-deficient mouse embryonic fibroblasts (MEF) exhibit delayed formation of filopodia and focal adhesion complexes when freshly plated. BAG3-deficient MEFs show reduced cell motility in culture. We observed that endogenous BAG3 protein is highly expressed in many human epithelial cancer cell lines, especially adenocarcinomas. Gene transfer–mediated overexpression of BAG3 increased motility of Cos7 cell and several human cancer cell lines, including breast cancer MCF7 and prostate cancer DU145 and ALVA31 cell lines. Conversely, reduction of BAG3 protein by RNA interference (RNAi) decreased cell motility in four of four epithelial tumor lines tested. We observed an influence of BAG3 on cell adhesion in culture. In Cos7 kidney epithelial cells, BAG3 protein partially colocalizes with actin at the leading edge of migrating cells, wherein active actin polymerization and nucleation occur. RNAi-mediated reductions in BAG3 expression were associated with decreased Rac1 activity, suggesting a role for BAG3 in regulating this small GTPase involved in actin-cytoskeleton dynamics. In mice, RNAi-mediated reductions in BAG3 in a human tumor xenograft suppressed invasion and metastasis in vivo. Thus, the high levels of BAG3 protein seen in some epithelial cancer cell lines may be relevant to mechanisms of tumor invasion and metastasis. [Cancer Res 2007;67(21):10252–9]

Introduction

BAG family cochaperones contain an evolutionarily conserved domain that binds Hsp70/Hsc70 family chaperones. Hsp70/Hsc70 family proteins regulate a variety of cellular processes, including signal transduction, transcription, and protein degradation (reviewed in ref. 1). The human genome contains six independent BAG genes. The Hsp70/Hsc70-binding BAG domain is accompanied by a diversity of other protein domains, depending on the particular family member (2). BAG3 is unique among the BAG family proteins for the presence of a WW domain, which is located near its NH2 terminus. WW domains represent protein interaction modules that bind a proline-rich motif, XPPXY (3–5). Some WW domains recognize their peptide ligands in a phosphorylation-dependent manner. These domains have been identified in a variety of signal transduction proteins that interact with plasma membrane receptor complexes or with components of the submembranous cytoskeleton (reviewed in refs. 3, 5). Thus, the presence of a WW domain suggests that BAG3 may target Hsc70/Hsp70 to specific proteins associated with receptors or involved in cytoskeleton regulation. BAG3 also contains a proline-rich region within which are found several SH3 ligand motifs, PXYP (6).

BAG3 was also reported independently to be the same as a protein called CAI stress response-1 (CAIR-1; ref. 6). BAG3/CAIR-1 expression is reportedly induced in A2058 human melanoma cells by exposure to CAI, an inhibitor of non–voltage-gated calcium channels. BAG3/CAIR-1 also forms complexes with Hsc70/Hsp70 and latent phospholipase Cγ (PLCγ) after stimulation of cells with epidermal growth factor (EGF). Because CAI causes tumor and endothelial cell cytostasis and inhibits cell attachment, migration, and angiogenesis, the authors speculated that BAG3 functions as a modulator of a cell growth or adhesion pathway involving PLCγ.

Gene ablation studies in mice show that BAG3 is required for postnatal survival of skeletal muscle myofibers, with BAG3-deficient mice dying a few weeks after birth of fulminant myopathy. Death of muscle is preceded by disorganization of the myosin contractile units, suggesting a role for BAG3 in homeostasis of cytoskeletal elements involved in muscle function (7). In muscle, BAG3 colocalizes with α-actinin on Z bands. The cell death and muscle degeneration that occurs in bag3−/− mice occurs after ultrastructural deterioration of the myosin-containing contractile units, suggesting it is a secondary consequence of dysregulation of some aspect of cytoskeletal function.

In this report, we explored the function of the BAG3 protein with a focus on its role in cell motility and adhesion. Our findings suggest that BAG3 localizes to the leading edge of migrating cells and that it regulates the actin-cytoskeleton, controlling cell motility, cell adhesion, and Rac activity. We also show that BAG3 is highly expressed in many human tumor cell lines, especially adenocarcinomas, and show that reduction of BAG3 inhibits invasive and metastatic activity of an epithelial cancer cell line in vivo, suggesting a contribution of BAG3 to the invasive or metastatic phenotype of cancers.

Materials and Methods

Cell culture and transfection. Low passage Cos7 cells were cultured in DMEM medium with 10% FCS with penicillin/streptomycin. Transfections were done with Lipofectamin reagent (Invitrogen). Mouse embryonic fibroblast (MEF) cells were prepared from E14.5 embryos by standard methods (8).

Gene silencing using short hairpin RNA vector. Gene silencing vector (pLTRH1) specific for mouse bag3 was generated, as described previously (7). For bag3 knockdown, virus-containing supernatant was added to Cos7, ALVA31, DU145, and MCF7 cells at a 1:5 ratio in DMEM 10% FCS. For stable

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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cell lines, transfected cells were selected using 1 μg/mL of puromycin. Either transient or permanent cell lines were used for cell motility analysis. For tumor xenograft experiments, A431 human prostate cancer cells were transfected with modified LTRH1 puro short hairpin RNA (shRNA) BAG3 vector or empty control vector, which has deleted 5′ long terminal repeat and packaging signal sequence to avoid making replication competent retrovirus. Two different subclones with >50% BAG3 protein knockdown were used for xenograft experiments.

**Cell motility and invasion assay.** Cos7 cells were transfected by pEGFP-BAG3, pcDNA3-FAK, and control plasmid with pEGFP vector and used for motility assays, as previously described (9). Cells (5 × 10⁴) were transferred to the upper chamber of transwell chambers (Costar, 8-μm pore size, 24 wells), which were pretreated with 10 μg/mL of fibronectin. After 8 h incubation at 37°C, the lower membrane was recovered and green fluorescent protein (GFP)-positive cells were counted in six fields at 10× magnification using fluorescent microscopy (Olympus BX60, Spot software). Similar experiments were done using shRNA gene silencing cell lines described above. For MEF cells, we used 5 h serum starvation before transwell motility assay. Cell viability was assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining, counting the percentage of cells with apoptotic nuclear morphology as described previously (7). For invasion assay, transwell chambers were coated on the upper surface with Matrigel (11 μg per filter; BD Biosciences).

**In vitro wound healing assay.** In vitro wound healing assays were carried out as another means of investigating cell migration. When confluence reached 95% to 100%, a wound was created in the center of the cell monolayer by gently removing the attached cells with a sterile plastic pipette tip. Cells that migrated into the wounded area were visualized and photographed under an inverted microscope.

**Adhesion assay.** For adhesion assays, 96-well, flat-bottomed plastic plates were coated with 10 μg/mL fibronectin (Sigma) or 0.01% poly t-ornithine (Sigma). Cells cultured in DMEM containing 0.5% fetal bovine serum for 16 h were plated at 2 × 10⁴ cells per well. Cells were allowed to adhere for 20 min at 37°C. At the end of incubation, the cells were fixed in 4% paraformaldehyde, then stained with 0.5% crystal violet for 30 min, followed by eluting dye with 1% SDS, and measured by absorption at 590 nm.

**Aptosis assay.** Apoptosis assays were done by staining cells with fluorochrome-conjugated Annexin V (Molecular Probes). Cells were incubated with Annexin V-conjugated Alexa Fluor 488 in Annexin-binding buffer for 15 min at room temperature and fixed. Apoptotic cell number was counted under fluorescent microscopy.

**Endogenous Rac1 activity assay.** After 5 h in serum-free media, cells were seeded onto fibronectin-coated plates (0.5 μg/mL Sigma) for 10 min and lysed in ice-cold Rac1 assay buffer [25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% v/v NP-40, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 10% glycerol, protease inhibitor]. For positive and negative controls, cells were incubated with 100 μmol/L GTPγS or 1 mmol/L GTP, respectively. After incubation with glutathione S-transferase (GST)-PAK-1 that binds the GTP-bound form of Rac1, Western blot was done by anti-Rac1 antibody (Pierce).

**Tumor xenograft experiments.** Cells were resuspended in 100 μL PBS, mixed with 100 μL of 1× Matrigel (BD Biosciences), and injected (2 × 10⁶ cells per injection) s.c. into the lateral flanks of male Nude-Foxn1 NU mice (Harlan). Tumor volumes were measured weekly, with calculation using this equation; volume = a × b × c × π / 6. After 9 weeks, all major organs, including primary tumor and lymph nodes, were fixed with 4% paraformaldehyde, followed by paraaffin embedding, and histologic analysis with 5-μm sectioning and H&E staining.

**Results**

**Delayed formation of filopodia and focal adhesion plaques in BAG3-deficient cells.** To explore the effect of the loss of BAG3 on actin cytoskeleton, we used murine cells in which the bag3 gene had been ablated (7). Embryos were collected at embryonic day E14.5 from bag3−/− brother-sister matings and fibroblasts cultures were prepared (8). Genotyping was accomplished by using DNA from yolk sacs of each embryo. For these experiments, MEF cells were plated on fibronectin-coated glass and fixed after 20, 40, or 60 min, followed by staining with the actin-binding fluorochrome, rhodamine-phalloidin. In contrast to bag3+/+ and bag3+/− cells, which formed filopodia within 20 min, most bag3−/−/MEFs were still lacking filopodia at 40 min, remaining mostly spheric, with a circumferential ring of actin (Fig. 1). By 60 min, however, bag3−/−/MEFs showed evidence of filopodia formation (Supplementary data), indicating a delay, but not an absence, of these cellular structures involved in cell migration. Quantifying the percentage of cells that have filopodia in freshly plated BAG3-deficient and wild-type MEF cell cultures confirmed a statistically significant delay in filopodia formation when BAG3 is lacking (Fig. 1B).

To further evaluate the effect of BAG3 deficiency on cell motility, we plated MEFs for 20 min, 60 min, or 24 h on fibronectin, then evaluated focal adhesion formation by staining with anti-Paxillin antibody (Fig. 1C). In bag3+/+ cells, within 20 min, Paxillin was localized to discrete foci at the cell periphery, consistent with rapid formation of focal adhesion plaques. In contrast, Paxillin was localized predominantly in a perinuclear pattern in bag3−/−/cells at 20 min after plating or fibronectin. At 60 min and 24 h, focal adhesion plaques had formed in bag3−/−/cells, indicating a delay, rather than a failure, to form focal adhesion plaques in cells lacking BAG3 protein (Supplementary data).

The differences in cell adhesion observed in bag3+/+ versus bag3−/− MEFs were not due to increased susceptibility of BAG3-deficient cells to cell death. Suspending MEFs by plating on polypropylene revealed similar cell death kinetics for bag3+/+ and bag3−/− cells (Supplementary data). Also, MEFs, as well as thymocytes from BAG3-deficient mice, did not show increased sensitivity to a variety of apoptosis-inducing agents (Supplementary data).

**BAG3-deficient MEFs have decreased cell motility.** We explored the effect of the loss of BAG3 on cell motility. For these experiments, transwell chambers were treated with 10 μg/mL of fibronectin and MEFs were placed into one side after 5 h of serum starvation, measuring migration to the opposite side. As shown in Fig. 1D, bag3−/− cells are less motile than bag3+/+ or bag3+/+ cells when cultured without serum. Under serum-depleted conditions, bag3−/− cells are unable to migrate, although they remained viable during the timeframe of these assays, excluding cell death as explanation for the findings. When cultured with serum, a difference in motility was not observed for bag3−/−, bag3−/+ and bag3−/+ MEF cells (not shown), suggesting alternative pathways for promoting cell migration are activated by serum growth factors.

**BAG3 is highly expressed in human cancer cell lines.** In preparation for evaluating the role of BAG3 in cancer cells, we analyzed expression of BAG3 protein in the National Cancer Institute's 60 human tumor cell line panel by immunoblotting (Fig. 2). An additional nine tumor lines were also included in this study. Lysates were normalized for total protein content (20 μg each) before SDS-PAGE/immunoblotting. BAG3-transfected 293T cells were also included as a positive control for all blots.

Relatively high levels of BAG3 were detected in ~16 of the 69 tumor lines (23%), based on comparisons with BAG3-transfected 293T cells or human B-cell leukemia cell line RS11846, which has high levels of endogenous BAG3. High levels of BAG3 were particularly prominent in adenocarcinomas, with BAG3 protein tending to be lower in squamous cell cancers, glioblastomas, melanomas,
and leukemias. Given that several of these tumors originated from tissues that do not seem to contain much BAG3 mRNA, this observation suggests that BAG3 expression may become abnormally up-regulated in association with carcinogenesis. Interestingly, comparison of 267B immortalized prostate cancer epithelial cells with the same cells stably transfected with oncogenic Ki-Ras suggests that activated Ras induces BAG3 protein expression (Fig. 2).

The predominant band seen in lysates was the expected ~80 kDa full-length BAG3 protein (Fig. 2). However, the “fuzzy” appearance of the band suggests that BAG3 is posttranslationally modified, consistent with a recent report of phosphorylation of BAG3 (6). Several tumor cell lysates also contained smaller molecular weight bands that were detected with the anti-BAG3 antibody, which we believe represent partial degradation products. Interestingly, however, one of the prostate cancer cell lines (LNCaP), two ovarian lines (OVCAR3, OVCAR8), two colon cancer lines (HCT116, KM12), a kidney line (ACHN), and two melanoma lines (SK-MEL2, UACC-62) contain an aberrant ~70-kDa immunoreactive band rather than the expected ~80-kDa full-length BAG3 protein. Some tumor lines contain both the normal p80 and the aberrant p70 band. Further work is required to determine the origin of this shorter form of BAG3 protein found in some cancer cell lines.

Overexpression of BAG3 increases cell motility. We explored the effects of BAG3 on motility of epithelial cancer cells. For these experiments, Cos7 cells were transiently transfected with pEFP control DNA or with a plasmid encoding BAG3, and migration of cells through a semipermeable membrane was assayed under serum-free conditions in transwell chambers (9). As a positive control, Cos7 cells were also transfected with a plasmid encoding FAK. Figure 3A shows a comparison of motility of Cos7 cells transfected with pEFP control DNA, BAG3-encoding plasmid, or FAK plasmid. Overexpression of BAG3 in Cos7 cells increased cell motility to a similar extent as FAK, causing a nearly doubling of the number of cells that migrated across membranes within the time used for experiments. Similar transfection experiments were done for ALVA31, Du145, and MCF7 epithelial cancer cell lines. Overexpression of BAG3 increases motility in these cancer cell lines as well, although results were most striking for ALVA31 prostate cancer cells (Fig. 3A). Immunoblot analysis confirmed expression of the proteins (Supplementary data).

BAG3 reduction decreases cell motility. We evaluated the effects of reducing levels of endogenous BAG3 protein in epithelial cancer cell lines, using shRNA-mediated gene silencing. For these experiments, transwell chambers were treated with 10 μg/mL fibronectin. Cells (5 × 10^4) were serum starved for 5 h and transferred to the upper chamber with or without serum-containing media. Cells migrating across the porous membrane after 8 h were fixed and stained with DAPI, and then cell counts were done for 10 fields using ×10 objectives.

Figure 1. bag3−/− MEF cells display delayed filopodia formation, delayed focal adhesion complex, and decreased cell motility. A, cover glasses were coated with 10 μg/mL fibronectin. Cells (1 × 10^5) were serum starved and incubated on cover glasses without serum. Cells were fixed at 20 min, stained with rhodamine-phalloidin, and examined by fluorescence microscopy. Note that most homozygous MEF cells lack filopodia and have rounded shapes at 20 min but have formed filopodia at 40 min after plating. In contrast, bag3−/− cells show a defect in filopodia extension. B, filopodia-containing cells were counted for bag3−/− and bag3+/+ cells at 40 min. Columns, mean; bars, SD (counting 10 fields with a 20× objective). C, MEFs with bag3+/+ or bag3−/− genotype were plated on glass coverslips for 20 min, then fixed and stained with rhodamine-phalloidin and anti-Paxillin monoclonal antibody, which was detected with Alexa Fluor 488 secondary antibody. Cells were imaged by confocal microscopy: actin (left), Paxillin (middle), merged (right). Representative cells are shown at two magnifications. D, transwell chambers were coated with 10 μg/mL fibronectin. Cells (5 × 10^4) were serum starved for 5 h and transferred to the upper chamber with or without serum-containing media. Cells migrating across the porous membrane after 8 h were fixed and stained with DAPI, and then cell counts were done for 10 fields using ×10 objectives.
fibronectin and loaded with either control-transfected or shRNA-transfected cells. As shown in Fig. 3B, shRNA targeting BAG3 suppressed motility of Cos7, ALVA31, Du145, and MCF7 cells in the Transwell assay. Immunoblot analysis verified reduction of BAG3 protein (Supplementary data). Note that reduction of BAG3 had no influence on proliferation or survival of these tumor cell lines under these culture conditions (Supplementary data and data not shown).

As an alternative to the use of transwell chambers to measure cell migration, we also used an in vitro "wound healing" model, wherein cell monolayers are scraped and migration of cells into the denuded region of the plastic culture dish is monitored over time. Using ALVA31 control and BAG3 shRNA-infected cells, we observed delayed migration of BAG3-deficient cells into the cleared area when cells were cultured in low serum (Fig. 3C). Less striking differences in cell migration were observed in the wound healing model when cells were cultured in complete serum-containing medium (not shown). These results thus corroborate the findings obtained by the Transwell method.

Finally, experiments were done to compare the invasiveness of tumor cells, in which BAG3 levels were reduced by gene silencing. For these experiments, ALVA31 prostate cancer cells were stably infected with control or BAG3 shRNA retrovirus, then plated on matrigel layers, and then their rates of migration through this extracellular matrix material were compared. Decreased expression of endogenous BAG3 in ALVA31 prostate cancer cells significantly inhibited their ability to invade Matrigel compared with control ALVA31 cells (Fig. 3D). Taken together, the results presented in Fig. 3 suggest that BAG3 contributes to cancer cell mobility and invasiveness.

**BAG3 regulates cell adhesion.** Because cell adhesion influences cell motility, we compared the rate of attachment of suspended epithelial cancer cell lines, in which BAG3 protein levels had been experimentally manipulated. Adhesion to either fibronectin-coated (integrin-dependent ligand) or poly l-ornithine–coated (proteoglycan-dependent ligand) plastic was studied. In short-term adhesion assays (20 min), more BAG3-overexpressing Cos7 cells adhered to fibronectin-coated plates than control-transfected Cos7 cells (Fig. 4A). Conversely, fewer Cos7 cells treated with BAG3 shRNA adhered to either fibronectin or poly l-ornithine (Fig. 4A). Similarly, fewer ALVA31, Du145, and MCF7 cells, in which BAG3 was reduced by shRNA treatment, adhered to either fibronectin (Fig. 4B) or poly l-ornithine (Fig. 4D) compared with control-treated cells. We conclude therefore that BAG3 regulates the adhesiveness of epithelial cancer cells. In addition, MEF cells from bag3−/− mice were also used for adhesion assays, showing decreased adhesion on fibronectin-coated plates (Fig. 4C).

**BAG3 colocalizes with actin at the leading edge of migrating cells.** Cos7 monkey kidney epithelial cells are motile under routine culture conditions and exhibit motile morphologies on plastic, with leading and lagging edges. We used these cells to localize BAG3 protein in relation to the actin cytoskeleton. First, GFP-tagged BAG3 was localized in Cos7 cells transfected with a plasmid encoding GFP-BAG3 fusion protein. Cells were fixed and stained with fluorescent antibodies to actin. As shown in Fig. 4C, BAG3 was localized to the leading edge of migrating cells, colocalizing with actin filaments. These results suggest that BAG3 plays a role in cell motility by regulating cell adhesion and cytoskeletal dynamics.
with rhodamine-conjugated phalloidin, then analyzed by confocal microscopy. GFP-BAG3 was located diffusely through transfected cells but was concentrated at the leading edge, where it extensively colocalized with actin (Fig. 5A).

Next, we localized endogenous BAG3 protein by immunofluorescence using antipeptide polyclonal BAG3 antibody. As shown in Fig. 5B, BAG3 and actin colocalized at the leading edge of motile cells. In contrast, BAG3 did not colocalize with Paxillin in Cos7 cells (Supplementary data), suggesting that BAG3 is not a component of focal adhesion plaques. Thus, BAG3 protein seems to be concentrated and colocalized with actin at leading edge, the cellular structures wherein active actin polymerization and nucleation is regulated for directed cell migration.

**BAG3 deficiency results in reduced Rac1 activation.** The small GTP-binding protein Rac1 is a well-known regulator of actin cytoskeleton dynamics in the context of integrin signaling (reviewed in ref. 10). We compared the activity of Rac1 in control vector-containing and BAG3 shRNA–expressing ALVA31 prostate cancer cells after fibronectin attachment in the absence of serum. Cell lysates were prepared, followed by recovery of the GTP-bound form of Rac1 by absorption to immobilized GST-Pak fusion protein. For positive and negative controls, we used lysates incubated with nonhydrolyzable GTP (GTPγS) and GDP, respectively. Less active Rac1 was recovered from lysates of BAG3 shRNA–expressing compared with control ALVA31 cells (Fig. 5C). In contrast, GTPγS-treated lysates showed comparable total levels of Rac1 in both control and BAG3 shRNA–treated cells. We conclude, therefore, that endogenous BAG3 regulates Rac1 activity.

**Tumor xenograft experiments reveal role for BAG3 in tumor growth, invasion, and metastasis in vivo.** ALVA31 cells that had been stably transfected with BAG3 shRNA or control vector (Fig. 6A) were used for tumor xenograft studies. Athymic male mice were inoculated s.c. with 10^7 ALVA31 cells, and tumor growth was measured by external calipers. Tumors containing BAG3 shRNA grew considerably slower than control ALVA31 cells (Fig. 6B).

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**Figure 3.** BAG3 expression alters cell motility of epithelial cancer cell lines. A, BAG3 overexpression increases motility. Cos7, ALVA31, MCF7, and Du-145 cells were transfected with plasmids encoding pEGFP-BAG3 or pcDNA3 FAK, or with empty control pEGFP plasmid (C). At 24 h after transfection, serum was withdrawn and cells were cultured at 37°C for 16 h. Cells (5 × 10^6) were transferred to the upper chamber of transwell chambers (pore size, 8 μm), which were pretreated with 10 μg/mL of fibronectin. After 8 h incubation at 37°C, the lower membrane was recovered and stained with DAPI, and cells were counted. Columns, mean; bars, SD (n = 6). *P < 0.001; **P < 0.01. B, shRNA-mediated knockdown of BAG3 decreases motility of cancer cell lines. Cos7, ALVA31, MCF7, and Du145 cells were infected with shRNA-BAG3 retrovirus or control virus. 5 × 10^4 cells were transferred to the upper chamber of transwell chambers (pore size, 8 μm), which were pretreated with 10 μg/mL of fibronectin. After 8 h incubation at 37°C, the lower membrane was recovered and GFP-positive cells were counted. Columns, mean; bars, SD (n = 6). *P < 0.001; **P < 0.01. C, reduction of BAG3 decreases cancer cellular motility as measured with in vitro wound healing assay. ALVA31 cells infected with control or shRNA BAG3 virus were cultured to confluence in six-well plates and scratched with a pipette tip to form a uniform wound. Cultures were continued for the indicated times, then cells were fixed and stained with DAPI. Images were obtained using fluorescence microscopy with DIC filter. Images are representative of eight separate culture wounds from two separate experiments. D, BAG3 protein reduction in ALVA31 cells decreases invasiveness in Matrigel. ALVA31 cells infected with control or BAG3 shRNA virus were plated on a layer of Matrigel. Cells that migrated to the lower surface of the membrane were counted (five fields at 10× magnification). Columns, mean; bars, SD. Representative of three separate experiments.
To examine metastatic capability, xenograft-bearing mice were maintained until two halves of the mice containing ALVA31 control cells showed indications of axillary lymph node enlargement, then all mice were sacrificed and their tissues analyzed histologically. Axillary lymph nodes from four of four mice containing control ALVA31 cells showed metastatic tumor. Multiple lung metastases were also observed in one of these mice. Also of note, the primary tumor mass was severely adherent to abdominal wall in three of four mice bearing control ALVA31 tumor, showing invasion of tumor into muscle wall and connective tissue. In contrast, no metastases were observed in eight of eight mice injected with BAG3 shRNA–expressing ALVA31 cells. Moreover, the subcutaneous tumors were not adherent to the underlying connective tissue and musculature. Thus, at least for ALVA31 cells, endogenous BAG3 seems to contribute to tumor growth, invasion, and metastasis in vivo.

Discussion

In this report, we show a role for BAG3 in cell motility and cell adhesion. Overexpression of BAG3 increases motility of epithelial cells in culture, whereas shRNA-mediated reductions in BAG3 reduced cell motility. Cell motility consists three sequential processes: protrusion, adhesion, and traction. Protrusion is the leading edge of movement, and it is induced by directional extension of the actin cytoskeleton. The extension step is coupled with integrin-mediated cell adhesion, which mediates attachment to extracellular matrix in a process in which focal adhesion plaques form to link adhesion complexes to the actin cytoskeleton. The mechanism of traction is mostly unknown and induced by passive movement (stretching) from the forward end of movement, but dissociation of focal adhesions is also important for this process. During the traction step, adhesion molecules are internalized into the cell and transported to the leading edge for reuse for subsequent attachments. Therefore, the actin cytoskeleton and adhesion molecules cooperate to regulate cell movement (reviewed in ref. 11). BAG3 protein colocalizes with actin at the leading edge of migrating cells. Consequently, based on the motility changes of cells in which BAG3 protein levels were experimentally manipulated and the intracellular location of the BAG3 protein, we speculate that BAG3 regulates some component of the actin cytoskeleton involved in cell migration. Of note, the effects of BAG3 on cell motility were most striking when cells were cultured with low serum, suggesting that serum factors stimulate BAG3-independent pathways for cell motility that mask the influence of BAG3. Thus, presumably both BAG3-dependent and BAG3-independent pathways regulate cell motility.

BAG3 also shows effects of cell adhesion in cell culture assays. For example, overexpression of BAG3 protein increased, whereas shRNA-mediated reduction in BAG3 protein decreased cell adhesion on both fibronectin and poly L-ornitine. This result suggests that BAG3 somehow regulates both integrin-mediated and proteoglycan-mediated adhesion. Because BAG3 did not colocalize with focal adhesion plaques (using Paxillin as a marker of these multiprotein complexes; Supplementary Fig. S1), it is unlikely to affect adhesion through a direct effect on integrins. Given that the actin cytoskeleton attaches to the intracytoplasmic regions of both integrin and nonintegrin cell adhesion molecules, we speculate that the observed alteration in cell adhesion is a secondary consequence of BAG3’s effects on actin cytoskeleton regulation. Although the mechanism by which BAG3 regulates actin cytoskeleton requires further investigation, we identified a role for BAG3 in controlling activity of small GTPase Rac1. Thus, taken together, our results suggest that BAG3 is a regulator of cell motility, possibly through regulating actin polymerization at the leading edge of migrating cells and probably through direct or indirect effects on Rac1.

BAG3 is a member of a family of cochaperones, which contain a conserved ~80 amino acid domain near their COOH terminus that binds Hsp70/Hsc70 chaperones. In contrast, the upstream regions of these proteins are highly diverse, presumably providing each of these proteins with a unique role in cell biology. BAG family genes are found throughout evolution, including yeast, plants, and animals (reviewed in ref. 1). Hsp70/Hsc70 family proteins serve a wide variety of functions in cells, including modulating protein function through changes in conformation, promoting multiprotein complex assembly/disassembly, facilitating protein translocation across organelar membranes, and ensuring proper folding of nascent polypeptide chains during translation (reviewed in refs. 12–14). Hsc70/Hsp70 family proteins have been implicated...
in the control of diverse biochemical events, including activation of transcription factors, proteolysis, and protein phosphorylation. A wide variety of signal-transducing proteins of relevance to cancer have been reported to be regulated either directly by Hsp70/Hsc70 family molecular chaperones or indirectly through Hsp90/Hsp70 interactions, including the protein tyrosine kinase Src, the serine/threonine kinase Raf-1, the tumor suppressors p53 and WT-1, and several transcription factors, including many of the nuclear hormone receptors, such as the glucocorticoid receptor, estrogen receptor, and androgen receptor (reviewed in ref. 15). Therefore, it is likely that BAG3 regulates cell motility by recruiting Hsp70/Hsc70 to other BAG3-interacting proteins on the actin cytoskeleton and modulating Hsp70/Hsc70 chaperone activity.

It has been suggested that BAG3 may interact with PLCγ, and we have also obtained evidence that it can bind the SH3 domain of PLCγ, as well as a variety of additional SH3-containing proteins, including c-Abl (data not shown). Given that BAG3 contains multiple PXXP motifs, it is possible it binds multiple SH3 proteins, which may link BAG3 to the process of cell movement. Recently, c-Abl has been reported as a regulator of F-actin polymerization (16), which may be of particular interest given that BAG3 colocalizes with actin at the leading edge of migrating cells and controls cell motility. Further analysis of the physiologic relevant proteins that interact with the PXXP motifs of BAG3 may reveal the importance of SH3 proteins in BAG3-mediated regulation of cell motility. Additionally, BAG3 contains a WW domain, which could also allow it to interact with regulators of the cytoskeleton, by analogy to other WW-containing proteins, such as actin-binding protein synaptopodin and tight junction protein MAGI-1 (17). The observations reported here lay a foundation for future structure-function studies that will eventually reveal the domains of BAG3 that are required for proper cytoskeleton dynamics and cell motility.

In addition to Hsp70/Hsc70 binding, BAG3 was reported to be a Bcl-2–binding protein and called “Bis” for “Bcl-2 interacting death suppresser” (18). The protein was identified using a method similar to the technique we used for the original cloning of BAG1, in which recombinant Bcl-2 protein was used as a ligand to screen a β-phage expression library by filter (plaque)–binding assay (18). Previously, we determined that BAG1 requires Hsp70 or Hsc70 for association with this important antiapoptotic protein (19). In gene transfer experiments where BAG3 was overexpressed, BAG3/Bis reportedly displayed little or no antiapoptotic activity but synergized with overexpressed Bcl-2 in preventing Bax-induced and Fas-mediated apoptosis (18). Although a role for BAG3 in apoptosis control is possible, we hypothesize that it is a secondary consequence of other functions of BAG3 and not a direct effect. In this regard, our prior analysis of bag3−/− mice revealed a requirement for this gene for postnatal maintenance of skeletal and cardiac muscle (7). BAG3-deficient mice developed normally, but the sarcomeric structures of muscle became disorganized after birth, showing deterioration of Z discs and myofibrillar disorganization, which preceded muscle cell death. In skeletal muscle, BAG3 localizes principally to Z bands, colocalizing with α-actinin at these cytoskeletal elements. Thus, in muscle, as well as fibroblasts and epithelial cells (shown here), BAG3 targets cytoskeletal structures, not mitochondria or other internal organelles where Bcl-2 resides. Also, we have been unable to detect specific association of Bcl-2 with BAG3 by communoprecipitation or GST pull-down assays, correlating with the lack of evidence of colocalization of BAG3 and Bcl-2 in intact cells. Furthermore, in BAG3-deficient mice, changes in expression of Bcl-2 and several prominent apoptosis-regulating proteins examined were not detected (7), excluding an effect on Bcl-2 expression.

While this manuscript was in preparation, others reported an effect of BAG3 on cell motility (20), observing that BAG3 inhibits cell motility in MDA-MD435 tumor and presenting evidence that BAG3 inhibits FAK-dependent, integrin-mediated adhesion. Interestingly, the MDA-MD435 cell line has an abnormal chromosome 11, wherein the gene encoding Hsc70 resides (21). Recently, deletions or point mutations affecting the N-terminal region of Hsc70 where BAG family proteins interact have been found in breast cancer cell lines (22). Thus, while differences in the methodologies used may contribute, we speculate that the difference in the phenotype observed for BAG3 overexpression in MDA-MD435 cells analyzed by others, compared with Cos7, MCF7, Du145, and ALVA31

Figure 5. BAG3 localizes with actin and regulates activity of small GTPase, Rac1. A, BAG3 and actin are partially colocalized at filopodia. Cos7 cells were transfected with pEGFP-bag3 and pEGFP control plasmid. Cells were fixed and stained with rhodamine-phalloidin. GFP (green) and F-actin (red) were detected by confocal microscopy. Left, actin cells; middle, GFP BAG3; right, merged images. B, endogenous BAG3 and actin colocalize at leading edge. Cos7 cells were fixed and stained with rhodamine-phalloidin and anti-BAG3 rabbit antiserum, detected with Alexa Fluor 488 secondary antibody, then imaged by confocal microscopy: actin (left), BAG3 (middle), and merged images (right). Two representative images are shown. Additional examples are provided as Supplementary data. C, Rac1 activity in lysates prepared from vector-transfected and BAG3 shRNA–transfected ALVA31 cells was determined by pull-down assays using immobilized GST-Pak, analyzing eluted proteins by immunoblotting with Rac-1 antibody. As positive and negative controls, cell lysates were incubated with GTPγS or GDP, respectively. Immunoblotting was used to assess total Rac1 protein levels in cell lysates, using the same volume of lysate from control and BAG3 shRNA–expressing cells.
Figure 6. BAG3 regulates tumor growth, invasion, and metastasis in vivo. Stable clones of ALVA31 cells were generated containing control or BAG3 shRNA vectors. A, SDS-PAGE/immunoblot analysis of BAG3 knockdown (clones S1 and S2) and control ALVA31 cells was done using 20 μg lysate per lane and rabbit polyclonal anti-BAG3 antibody (top). The blot was reprobed with antiactin antibody (bottom). B, nude mice were injected s.c. with four control ALVA31 or BAG3 shRNA–expressing subclones S1 and S2. Tumor volume was determined by external caliper measurements. Statistical significance was determined by unpaired t test, revealing P < 0.05 for comparison of control with either S1 or S2 at all times >24 h. C, metastatic tumors observed in mice injected with control ALVA31 but not BAG3 knockdown cells. Photomicrographs of tissues from mice bearing control ALVA31 xenografts show multiple metastatic tumors in lung (left), lymph node metastasis (middle), and example of tumor invasion into abdominal wall (right).

cell analyzed here, may be attributable to differences in the status of Hsc70 in these tumor cell lines. Further examination of Hsc70 mutations and consideration of alternative explanations are required to clarify the underlying mechanisms responsible for the different phenotypic effects of BAG3 on motility in various epithelial cancer cell lines. It is also possible that massive overexpression of BAG3 may disrupt the stoichiometry of multiprotein complexes in which BAG3 participates, particularly given that BAG3 possesses several potential protein interaction domains and thus has characteristics of a scaffold protein. Thus, modest overexpression of BAG3 may promote cell motility, whereas massive overexpression may inhibit motility. The phenotypes reported here of tumor cell lines in which endogenous BAG3 was knocked down indicate that the predominant role of BAG3 is as an enhancer of cell motility rather than an inhibitor, at least in most tumor cell lines.

The finding that some cancers contain high levels of BAG3 raises the possibility that overexpression of this cell motility–regulating protein could contribute to tumor invasion or metastasis. In support of this hypothesis, we observed a requirement for endogenous BAG3 for invasion and metastasis of ALVA31 prostate cancer cells in tumor xenograft experiments using immunocompromised mouse recipients. Although preliminary, when taken together with the effects of BAG3 overexpression and knock down on epithelial cancer cell motility and adhesion in culture, these findings suggest that BAG3 regulates cellular phenotypes of relevance to tumor invasion and metastasis, warranting further investigation.

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