

# Bag1 Proteins Regulate Growth and Survival of ZR-75-1 Human Breast Cancer Cells<sup>1</sup>

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## ABSTRACT

Bag1 proteins bind heat shock protein  $M_r$  70,000 (Hsp 70) family molecular chaperones and regulate diverse pathways involved in cell proliferation, apoptosis, and stress responses. Four isoforms of Bag1 can be produced from a single gene in humans, including a nuclear-targeted long version (Bag1L) and a shorter cytosolic isoform (Bag1). Because overexpression of Bag1 and Bag1L has been reported in breast cancers, we explored the effects of Bag1 and Bag1L on the growth of ZR-75-1 human breast cancer cells cultured *in vitro* and in tumor xenograft models using immunocompromised mice. Cells stably transfected with expression plasmids encoding either Bag1 or Bag1L displayed comparable rates of growth in cultures containing 10% serum, compared with control-transfected ZR-75-1 cells. In contrast, ZR-75-1 cells stably expressing mutants of Bag1 or Bag1L, which lack the COOH-terminal domain ( $\Delta$ C) required for heat shock protein  $M_r$  70,000 binding, displayed retarded growth rates. When cultured without serum, the viability of control-transfected, as well as Bag1 $\Delta$ C- and Bag1L $\Delta$ C-expressing, cells declined with time, whereas Bag1- and Bag1L-overexpressing ZR-75-1 cells survived for over a week in culture. Caspase protease activation induced by serum deprivation was also prevented by stable expression of either Bag1 or Bag1L in ZR-75-1 cells. In addition, sensitivity to anchorage dependence was restored partially in ZR-75-1 cells expressing dominant-negative Bag1 $\Delta$ C and Bag1L $\Delta$ C. In tumor xenograft studies involving injection of ZR-75-1 cells into mammary fat pads of female *nu/nu* mice, ZR-75-1 cells expressing Bag1 or Bag1L formed 1.4–1.6-fold larger tumors compared with control-transfected cells, whereas tumors formed by Bag1 $\Delta$ C- and Bag1L $\Delta$ C-expressing cells grew very slowly and reached sizes < one-third of tumors generated by Neo-control ZR-75-1 cells. Altogether, these findings demonstrate that Bag1 and Bag1L provoke similar changes in breast cancer cell growth and survival and suggest that interference with Bag1 or Bag1L function might be a useful strategy for opposing breast cancer.

## INTRODUCTION

Bag-family proteins contain an evolutionarily conserved domain (the “BAG domain”) that binds with high affinity to the ATPase domain of Hsp70 family proteins,<sup>5</sup> modulating the activity of these molecular chaperones (reviewed in Ref. 1). In addition to Hsp70 binding, BAG-family proteins also interact with a variety of other proteins, potentially altering their activity directly or indirectly by targeting Hsp70 upon them. The founding member of this family, Bag1, was first identified based on its ability to associate with the antiapoptotic protein Bcl-2 and has been implicated in suppression of

apoptosis (2–7). However, Bag1 proteins have been implicated in a wide variety of cellular processes besides apoptosis, including regulation of cell proliferation, cell migration, and responses to stress (5, 8, 9).

Overexpression of Bag1 proteins has been documented in some types of human cancers, *e.g.*, pathological elevations in either cytosolic or nuclear Bag1 proteins have been reported in adenocarcinomas of the breast cancers and squamous cell carcinomas of the oral cavity, correlating with differences in patient survival (10–12). In this regard, the human *BAG1* gene is capable of encoding as many as four different isoforms of the Bag1 protein, through a mechanism involving usage of alternative translation initiation codons in a single mRNA (13–16). The most abundant of these is p36 Bag1, which resides predominantly in the cytosol. In contrast, the p50 Bag1L protein contains an additional NH<sub>2</sub>-terminal domain, which contains candidate nuclear localization signal sequences and has been shown to be a nuclear protein exclusively (14–17). Although additional isoforms of Bag1 protein can arise in humans, including p46 Bag1M (Rap46), these have not been observed in mice or other species, and they tend to be present at lower levels than p36 Bag1 or its equivalent in the mouse (13–16).

The cytosolic p36 Bag1 and nuclear p50 Bag1L proteins presumably interact with different target proteins in cells, given the differences in their location. Supporting this concept, data have been presented indicating that nuclear isoforms of Bag1 can modulate the activity of several transcription factors, whereas cytosolic Bag1 does not (6, 13, 18–20). Conversely, cytosolic p36 human Bag1 (or p29 mouse Bag1) has been implicated in the regulation of several cytosolic proteins (*e.g.*, Bcl-2, epidermal growth factor receptor, and hepatocyte growth factor receptor) with which nuclear isoforms of Bag1 presumably would not come into contact (2, 21). Still, other identified Bag1-binding proteins, such as Siah1 and Raf1, can shuttle between cytosol and nucleus and therefore might be relevant to both cytosolic and nuclear isoforms of Bag1 protein (8, 22, 23).

Because abnormal elevations in the expression of cytosolic and nuclear Bag1 proteins have been observed in breast cancers (10, 11), we explored the effects of overexpressing either p36 Bag1 or p50 Bag1L on the growth of a human breast cancer cell line ZR-75-1 in culture and when implanted orthotopically into the mammary fat pads of immunocompromised female mice. Comparisons were also made with ZR-75-1 cells expressing dominant-negative mutants of Bag1 and Bag1L, which lack the COOH-terminal “BAG” domain required for Hsp70 binding (19, 23–25). These studies provide the first analysis of the functions of Bag1 proteins in breast cancers, providing evidence that both Bag1 and Bag1L can regulate breast tumor growth *in vivo*. The similar phenotypes observed for ZR-75-1 cells expressing either Bag1 or Bag1L (and for ZR-75-1 cells expressing Bag1 $\Delta$ C versus Bag1L $\Delta$ C) may have important implications for understanding the molecular mechanisms by which cytosolic and nuclear isoforms of Bag1 modulate the growth and survival of cancer cells.

## MATERIALS AND METHODS

**Transfections.** The plasmids pcDNA3-Bag1L, pcDNA3-Bag1L $\Delta$ C, pcDNA3-Bag1, and pcDNA3-Bag1 $\Delta$ C have been described previously (Ref.

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<sup>5</sup> The abbreviations used are: Hsp70,  $M_r$  70,000 heat shock protein; Bag1L, Bag1-Long; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfonyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; Poly-HEMA, Poly(2-hydroxyethyl methacrylate); AFC, amino-4-trifluoromethyl-coumerin; Ac-DEVD, acetyl-Aspartyl-L-Glutamyl-Valinyl-Aspartyl; ER, estrogen receptor.

19; available from ScienceReagents, Inc., Atlanta, GA). The breast cancer ZR-75-1 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in RPMI 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% FCS, 3 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin ("Complete Medium"). For stable transfections, 12 µl of Fugene (Roche, Indianapolis, IN) was diluted in 88 µl of OptiMEM (Invitrogen) and then added to 2 µg of plasmid DNA. This lipid-DNA complex was then added to the media covering 60-mm dishes (Costar, Corning, NY) containing ZR-75-1 cells at ~50% confluence. Two days after transfection, the cells were recovered from dishes by trypsinization and resuspended in complete medium containing 1 mg/ml G418 (Life Technologies, Inc., Gaithersburg, MD), and 100 cells were seeded per well into 96-well, flat-bottomed plates (Costar). During selection, the cells were fed with complete media containing 1 mg/ml G418. Wells containing colonies were expanded and analyzed for expression of Bag1 by immunoblotting.

**Cell Growth Assays.** Cells were resuspended in complete medium and plated at 100 µl/well into 96-well, flat-bottomed plates at an initial density of 5 × 10<sup>3</sup> cells/well and allowed to adhere for 24 h. Cells were then cultured for various times in complete medium or medium without serum before adding 50 µl/well of 1 mg/ml XTT (Polysciences, Inc., Warrington, PA) in RPMI 1640 containing 25 µM phenazine methosulfate. After culturing for 1 or 3 h at 37°C and then agitating plates gently for 5 min, the absorbance was read at 450 nm using a 96-well plate reader (Powerwave × 340; Bio-Tek Instruments, Inc., Summit, NJ). Pilot experiments verified that the cell densities encountered in these experiments were within the linear portion of the XTT assay. All assays were performed in triplicate and mean ±SE was calculated. A minimum of three independent experiments was performed.

**Anchorage-independent Cell Culture.** Flat-bottomed, 96-well plates were coated with Poly-HEMA by applying 50 µl/well of a 10 mg/ml solution of polyhydroxyethylmethacrylate in ethanol, air drying, and repeating the treatment, followed by three washes in PBS (pH 7.4; Ref. 26). Cells were plated into poly-HEMA-coated, 96-well plates at an initial density of 5 × 10<sup>3</sup> cells/well in RPMI 1640, supplemented with 10% FCS and cultured for 0–4 days. Relative numbers of viable cells were compared by XTT assay. Pilot experiments in which trypan blue dye exclusion was used as an alternative method for assessing cell viability confirmed the validity of the XTT assay results.

**Caspase Activity Assays.** Cells were plated into 6-well plates at an initial density of 1 × 10<sup>6</sup> cells/well and allowed to adhere for 24 h in complete medium. The following day, cells were cultured either with medium containing or lacking serum. At various times thereafter, the cells were collected by trypsinization and lysed in 10 mM Tris (pH 7.3) containing 25 mM NaCl, 0.25% Triton X-100, and 1 mM EDTA. Caspase activity was assayed by release of AFC from Ac-DEVD-AFC substrate peptide (1 nM final concentration; Calbiochem, La Jolla, CA), using a fluorimeter (Perkin-Elmer LS50B) equipped with a thermostated plate reader, as described (27, 28).

**Immunoblot Analysis.** Cells were collected and washed twice with ice-cold PBS (pH 7.4). Cell pellets were lysed in radioimmunoprecipitation assay buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% Na deoxycolate, 0.1% SDS, and 5 mM EDTA] containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and Complete Protease Inhibitor; Roche). Aliquots containing 20 µg of protein were subjected to SDS-PAGE (10% gels), followed by electrotransfer to nitrocellulose (0.45 µm) membranes (Millipore Corp., Bedford, MA). Bag1 proteins were detected using the KS6C8 monoclonal anti-BAG-1 antibody (Ref. 15; available from DAKO, Inc, Carpinteria, CA) followed by horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Bound antibodies were visualized using an enhanced chemiluminescence detection method (Amersham Pharmacia Biotech).

**Tumor Xenograft Studies.** Six-week-old ovariectomized *nu/nu* female mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were implanted s.c. with an estrogen-release pellet (60-day release pellet containing 0.72 mg of estradiol; Innovative Research of America, Sarasota, FL) under anesthetic conditions using 0.015 ml/gram AVERTIN (i.p.). Control animals were subjected to the same procedures, but pellet implantation was omitted. Two days after surgery, exponentially growing ZR-75-1 cells expressing Neo, Bag1, Bag1L, Bag1ΔC, or Bag1LΔC were detached with trypsin. The trypsin was neutralized with medium containing serum, and the cells were washed twice by centrifur-

gation, counted, and resuspended in serum-free RPMI 1640 at 3 × 10<sup>7</sup>/ml. Each animal received two injections of tumor cells, one on each side, in the mammary fat pads between the first and second nipples. Primary tumor growth was assessed by measuring the external volume of each tumor every 3–4 days using calipers, measuring three mutually perpendicular diameters (mm). The geometric mean diameter was then used to calculate the tumor volume in mm<sup>3</sup> using the equation  $V = (1/6)[\pi](d1d2d3)$ . At the end of the experiment, the animals were sacrificed by CO<sub>2</sub> asphyxiation. Statistical analysis of data were performed using a one-sided ANOVA or unpaired *t* test.

## RESULTS

To contrast the effects of Bag1 and Bag1L on the growth properties of breast cancer cells, the ER-positive tumor line ZR-75-1 was transfected with plasmids encoding either Bag1 or Bag1L or with control plasmid ("Neo"), and stable transfectants were selected in G418. Plasmids encoding mutants of BAG1 and BAG1L that lack the COOH-terminal BAG domain required for Hsc70/Hsp70 binding were also transfected (19, 23–25). Previous studies have demonstrated the faithful targeting of the Bag1L and Bag1LΔC proteins to nuclei and have confirmed a cytosolic location of Bag1 and Bag1ΔC (17). Several independent G418-resistant clones were isolated and analyzed by immunoblotting for expression of Bag1 proteins. Fig. 1 shows representative results from some of the clones used for this study. Untransfected and Neo-control transfected ZR-75-1 cells express the three major isoforms of BAG1, including p36 Bag1, p46 Bag1M, and p50 Bag1L. Clones of stably transfected ZR-75-1 cells were identified, which contained 5–10-fold elevations in these proteins (Fig. 1). In contrast, the Bag1ΔC and Bag1LΔC proteins were produced at much lower levels, consistent with prior reports suggesting that accumulation of these proteins may be limited by instability of these truncated proteins (8, 17, 19, 20, 24). Immunoblotting analysis permitted comparison of the relative levels of the Bag1ΔC and Bag1LΔC proteins with the endogenous Bag1 and Bag1L proteins, respectively.

When growth was assessed under routine culture conditions (10%

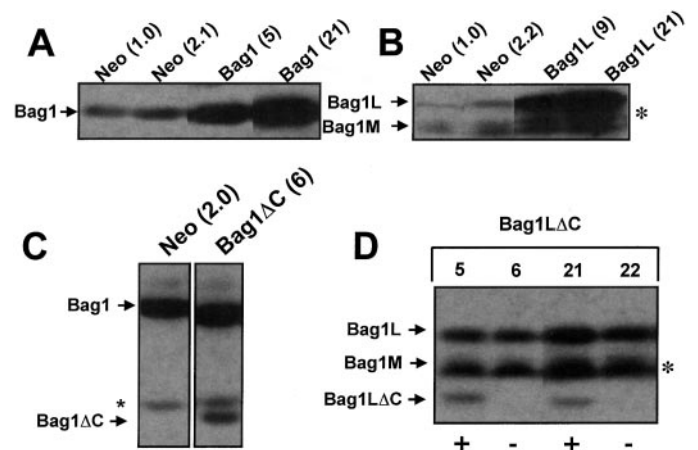
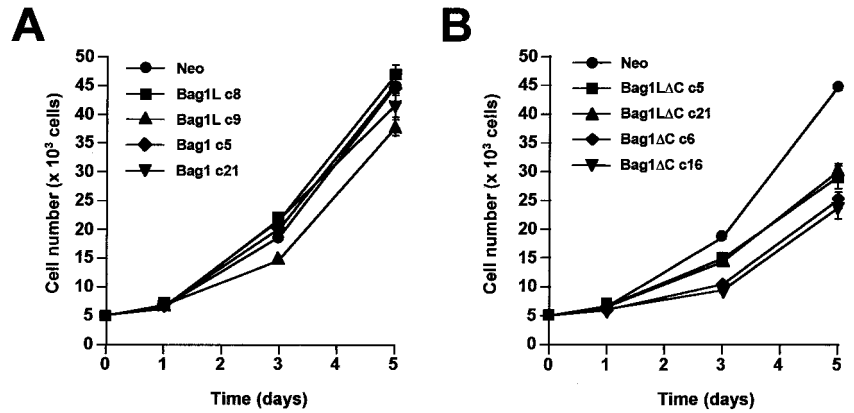


Fig. 1. Immunoblot analysis of BAG1 transfectants. ZR-75-1 cells were stably transfected with plasmids encoding Bag1, Bag1L, COOH-terminal deletion mutants of these proteins (ΔC), or control pcDNA3 (*Neo*) plasmid. Immunoblot analysis of lysates (20 µg/lane) was performed using anti-Bag1 monoclonal antibody and an enhanced chemiluminescence-based detection method. Data from several independent G418-resistant clones are presented (clone numbers indicated in parentheses). Reprobing blots with an antibody to β-tubulin confirmed loading of equivalent amounts of total protein in each lane (data not shown). The positions of the p36 Bag1, p46 Bag1M, and p50 Bag1L protein are indicated by arrows (left). Additional bands recognized by the anti-BAG1 representing post-translational modification of the Bag1M protein (Ref. 18; B and D) or a cross-reactive band (C) are indicated by \*. In D, the relative levels of endogenous Bag1L are compared with transgene-derived Bag1LΔC, showing examples of clones (5 and 21) that expressed Bag1LΔC (+) and other clones that did not (clones 6 and 22; -). Some of the lanes on blots were resequenced for clarity of presentation. Data are representative of several independent clones used for subsequent studies.

Fig. 2. Overexpression of Bag1 or Bag1L COOH-terminal deletion mutants slows the growth rate of cells in culture. Clones of stably transfected ZR-75-1 cells which contained Neo-control plasmid or plasmids producing either wild-type Bag1 or Bag1L (A) or dominant-negative mutants Bag1ΔC or Bag1LΔC (B) were plated into 96-well plates in RPMI 1640, supplemented with 10% FBS, and the growth of the cells was followed by XTT assay (mean ±SE; n = 3) on days 0, 1, 3, and 5. Relative numbers of viable cells were estimated based on comparisons to a standard curve. The clone numbers for the stable transfectants are indicated.



serum), no significant differences were evident in the rate of cell accumulation in cultures of ZR-75-1 cells transfected with control plasmid compared with plasmids producing Bag1 or Bag1L (Fig. 2A). In contrast, clones of ZR-75-1 cells stably transfected with plasmids encoding either the Bag1ΔC or Bag1LΔC mutant proteins displayed slower growth rates in culture (Fig. 2B). Similar results were obtained for two of two independent transfected clones tested for both Bag1ΔC and Bag1LΔC. These results suggest that the Bag1ΔC and Bag1LΔC proteins exert a dominant-negative effect, consistent with prior reports where activity of such truncation mutants has been assessed in other contexts (8, 17, 19, 20).

**Overexpression of Either BAG1 or BAG1L Enhances Survival of Serum-deprived ZR-75-1 Breast Cancer Cells.** Because no significant differences were evident in the rate of cell accumulation in cultures of ZR-75-1 cells overexpressing wild-type Bag1 or Bag1L when assayed under routine culture conditions, we explored whether differences in the growth properties of the Bag1 and Bag1L transfectants could be demonstrated under suboptimal circumstances. To this end, stable transfectants of ZR-75-1 cells were cultured in serum-deficient medium, and the relative number of

viable cells was determined at various times thereafter by XTT assay. ZR-75-1 cells overexpressing either Bag1 or Bag1L were capable of prolonged survival in the absence of serum, in contrast to Neo-control transfectants, which died in a time-dependent manner (Fig. 2A). This cell death was caused by apoptosis, as determined by microscopic visualization of cells (data not shown) and by caspase activity assays, which demonstrated marked increases in Ac-DEVD-AFC hydrolytic activity in serum-deprived Neo-control transfected cells but not in ZR-75-1 cells overexpressing Bag1 or Bag1L (Fig. 2B). Similar results were obtained for three of three stably transfected clones of Bag1 and Bag1L overexpressing ZR-75-1 cells (data not shown). In contrast, the rate of cell death and the extent of caspase activation in cultures of serum-deprived ZR-75-1 cells expressing either the Bag1ΔC or Bag1LΔC mutant proteins were not different significantly from control-transfected cells (Fig. 3, C and D). Thus, whereas Bag1ΔC and Bag1LΔC slow the rate of growth of ZR-75-1 cells in serum-containing medium, these mutant proteins do not appear to accelerate cell death induced by serum deprivation.

Fig. 3. Overexpression of Bag1 or Bag1L enhances cell survival in the absence of serum. Clones of stably transfected ZR-75-1 cells were plated in RPMI 1640, supplemented with 10% FBS, and allowed to adhere for 24 h in 96-well, flat-bottomed plates (5 × 10<sup>3</sup> cells/well). The medium was then replaced with RPMI 1640-lacking serum, and the relative number of viable cells was monitored by XTT assay at days 0, 1, 3, 5, 7, and 9 (A and C). Cell lysates were also prepared from serum-starved cells cultured in the same manner and assayed for the presence of active caspases, using a fluorogenic substrate Ac-DEVD-7-amino-4-trifluoromethyl coumann (B and D). Results are expressed as the mean ±SE (n = 3). The results in A and C were confirmed by trypan blue dye exclusion assays (data not shown).

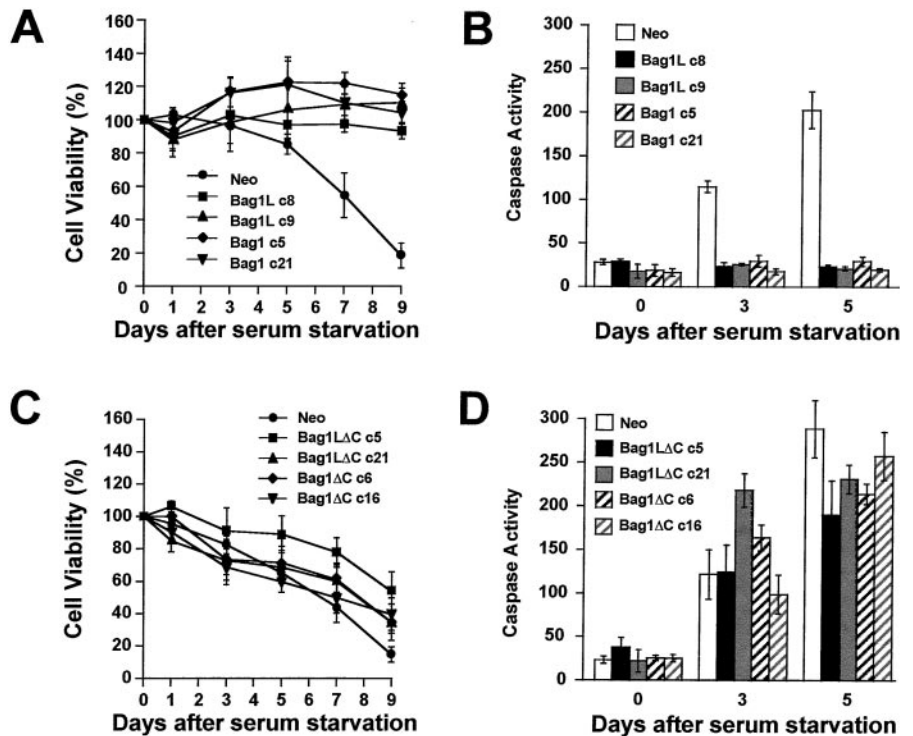
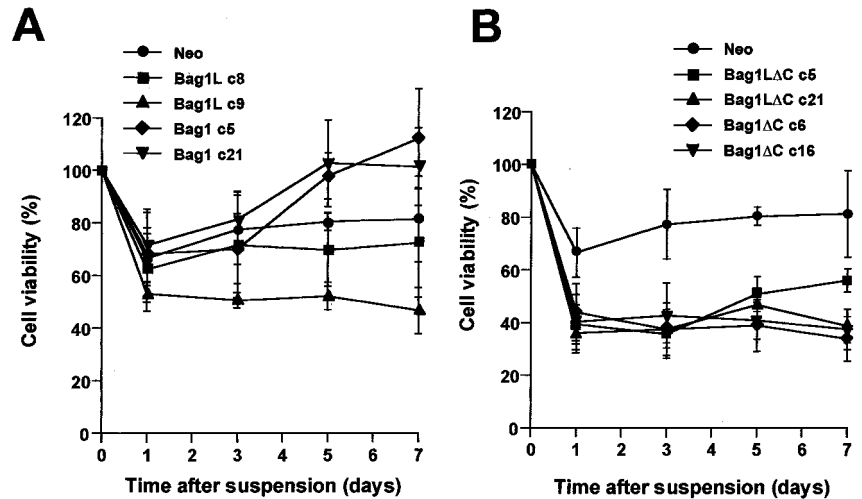


Fig. 4. Overexpression of either Bag1ΔC or Bag1LΔC restores anchorage dependence of ZR-75-1 cells. Clones of stably transfected ZR-75-1 cells were transferred into poly-HEMA-coated, 96-well plates ( $5 \times 10^3$  cells/well), and cell viability was monitored by XTT assay (mean  $\pm$ SE,  $n = 3$ ) at various days thereafter.

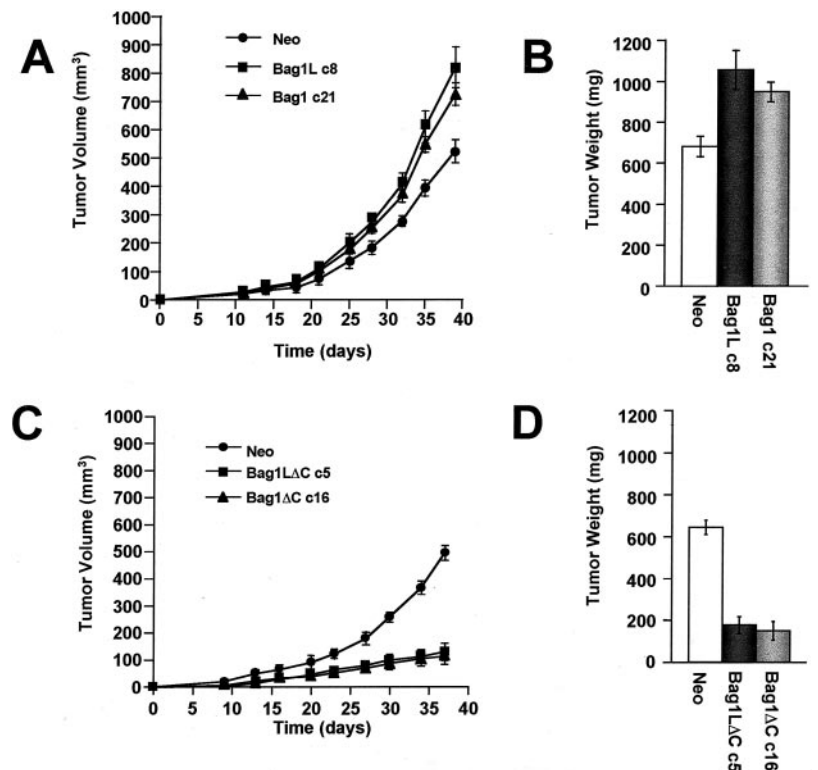


**Bag1ΔC and Bag1LΔC Restore Anchorage Dependence of ZR-75-1 Cells.** Tumor cells are well known for their ability to grow and survive in an anchorage-independent manner, in contrast to normal epithelial cells which undergo apoptosis when deprived of attachment to extracellular matrix proteins (reviewed in Ref. 29). Therefore, we contrasted the survival of the various stable transfectants of ZR-75-1 cells when plated on poly-HEMA-coated plates, which prevents cell attachment (26). Parental and Neo-control transfected ZR-75-1 cells survived detachment, with relative numbers of viable cells remaining near their starting levels for  $\leq 7$  days, following a transient decline in XTT dye reduction associated with the initial detachment (Fig. 4A). In contrast to ZR-75-1 cells overexpressing wild-type Bag1 or Bag1L, breast cancer cells expressing the mutant Bag1ΔC or Bag1LΔC proteins displayed increased sensitivity to detachment, with over half the cells dying as determined by both XTT dye reduction assays (Fig. 4B) and trypan blue dye exclusion assays (data not shown). Although

substantial inter and intraclonal variation was observed among stable transfectants under anchorage-independent culture conditions, the clones expressing Bag1ΔC or Bag1LΔC reproducibly exhibited greater dependence on anchorage than Neo-control transfectants in four of four experiments involving side-by-side comparisons ( $P < 0.00008$  by one-way ANOVA).

**Comparisons of Growth of ZR-75-1 Transfectants *in Vivo*.** Various stable transfectants of ZR-75-1 were used for tumor xenograft studies in *nu/nu* mice. After injection of  $1.5 \times 10^6$  tumor cells into the fat pads of female mice, the rate of tumor formation was compared for ZR-75-1 cells transfected with Neo-control plasmid *versus* plasmids encoding Bag1, Bag1L, Bag1ΔC, or Bag1LΔC. Compared with control ZR-75-1 cells, tumor cells overexpressing Bag1 or Bag1L demonstrated enhanced growth in mice, forming larger volume tumors than Neo-control cells (Fig. 5, A and B). The onset of palpable tumors was not significantly different for the Bag1- and Bag1L-expressing

Fig. 5. Bag1 and Bag1L enhance growth of ZR-75-1 tumors in mice. Clones of stably transfected ZR-75-1 cells were injected ( $1.5 \times 10^6$  cells) into the mammary fat pads of female *nu/nu* mice. The growth of tumors was followed by measuring the external volume using calipers (A and C). The weight of tumors at the end of the experiment was also determined (B and D). Data represent mean  $\pm$ SE ( $n = 8-9$ ). The difference in tumor weight was statistically significant, comparing Neo-Controls to either Bag1 ( $P < 0.01$ ) or Bag1L ( $P < 0.01$ ; B) and comparing Neo-Control to Bag1ΔC ( $P < 0.001$ ) and Bag1LΔC ( $P < 0.001$ ), as determined by one-way ANOVA.



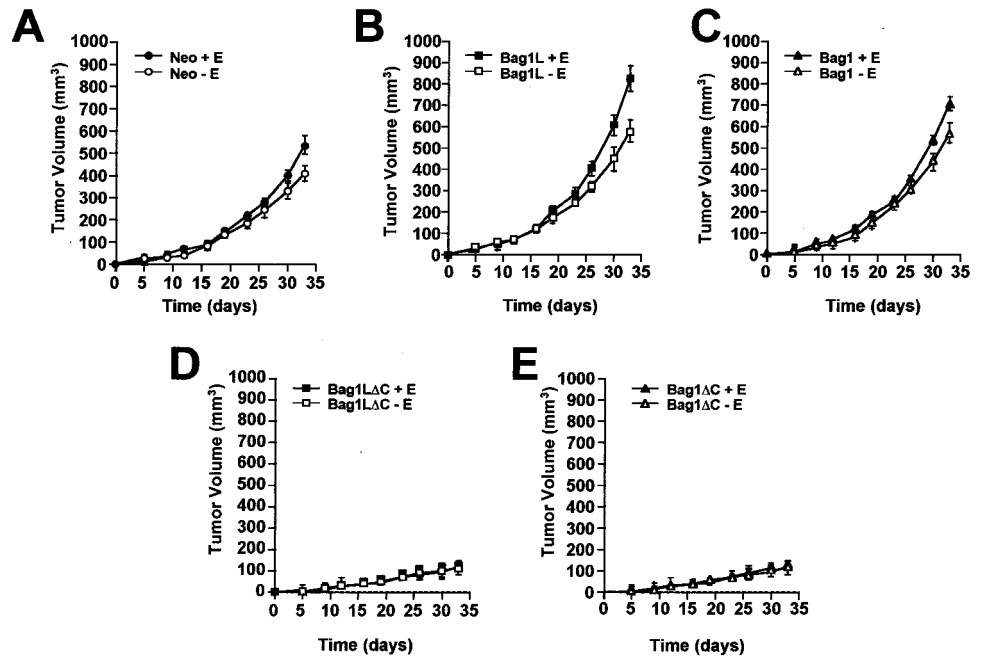


Fig. 6. Effects of estrogen on growth of control and genetically modified ZR-75-1 tumors in mice. Ovariectomized female *nu/nu* mice were implanted s.c. with an estrogen-release pellet (black symbols) or sham operated (white symbols). Two days later, clones of stably transfected ZR-75-1 cell lines were injected ( $1.5 \times 10^6$  cells) into the mammary fat pads of the mice, and the volume of the tumor was monitored by external measurements using calipers. Data represent mean  $\pm$  SE ( $n = 6$ ). ZR-75-1 cells tested included clones stably transfected with Neo-control plasmid (A) or plasmids encoding Bag1, Bag1L, Bag1ΔC, or Bag1LΔC (B–D), as indicated.

clones compared with Neo-control cells, but the size of the tumors began to diverge at  $\sim 3$  weeks after injection and continued until animals were sacrificed at 5–6 weeks because of tumor burden ( $P < 0.01$ ). Histological and immunohistochemical analysis of the tumors confirmed the presence of adenocarcinomas, which expressed human markers typical of ZR-75-1 cells (data not shown). In contrast to tumor cells expressing wild-type Bag1 or Bag1L, ZR-75-1 cell clones expressing the Bag1ΔC or Bag1LΔC dominant-negative proteins displayed markedly retarded rates of growth in mice (Fig. 5, C and D;  $P < 0.001$ ). Although we cannot exclude the possibility that ZR-75-1 cells expressing Bag1(ΔC) or Bag1L(ΔC) could eventually form full-sized tumors given sufficient time, during the 7 weeks of follow-up time of our studies, the weights of these tumors never was  $>200$  mg, compared with 600–700 mg for tumors generated with Neo-control ZR-75-1 cells and 900–1100 mg of tumors for Bag1 and Bag1L overexpressing ZR-75-1 cells (Fig. 5).

Because ZR-75-1 expresses ERs and is estrogen responsive, we also contrasted the rates of tumor growth in oophorectomized female *nu/nu* mice implanted with estrogen-releasing *versus* control pellets. The loss of estrogen slowed the rates of growth in mice of Neo-control, Bag1, and Bag1L breast cancer cells to comparable extents (Fig. 6, A–C), implying that overexpression of Bag1 or Bag1L does not negate the growth advantage provided by estrogen. In contrast, the presence or absence of estrogen had no detectable difference on the slow growth rate of ZR-75-1 cells expressing the Bag1ΔC or Bag1LΔC dominant-negative proteins (Fig. 6, D and E).

## DISCUSSION

The findings presented here provide evidence that both cytosolic p36 Bag1 and nuclear p50 Bag1L can have potent effects on the regulation of breast cancer cell growth *in vitro* and *in vivo*. Specifically, using the human breast cancer cell line ZR-75-1 as a model, evidence was obtained that elevated levels of either Bag1 or Bag1L can enhance survival during growth factor deprivation and accelerate the rates of tumor growth *in vivo*, whereas mutants of these proteins lacking the conserved Hsp70-binding domain slow growth rates *in vitro*, partially restore sensitivity to anchorage, and profoundly reduce rates of tumor growth in mice.

ZR-75-1 cells were chosen for these studies in part because they express ERs and are estrogen responsive. At least one isoform of Bag1 (p46 Bag1M/RAP46) has been reported to associate with ER *in vitro* (13), and nuclear Bag1L has been reported to enhance the transcriptional activity of some steroid hormone receptors (androgen, progesterone, and vitamin D3), whereas the glucocorticoid receptor can be repressed by certain Bag1 proteins (6, 18–20). Thus, it was of interest to contrast the effects of cytosolic Bag1 with nuclear Bag1L in tumor cells that express a steroid hormone receptor known to be of clinical relevance to breast cancer (*i.e.*, ER).

Bag1 and Bag1L conferred similar phenotypes with respect to growth and survival of ZR-75-1 cells, suggesting that the targets of these Hsp70 regulators may be similar, despite the different intracellular locations of these proteins. In this regard, Bag1 proteins have been reported to associate directly or indirectly with multiple other proteins, making it difficult to ascribe their actions to a single target or even a single pathway (reviewed in Ref. 1). Thus, whereas the similar phenotypic consequences of overexpressing p36 Bag1 and p50 Bag1L (or expressing Bag1ΔC *versus* Bag1LΔC mutants) imply that the relevant target proteins may be the same, it is also possible that different target proteins and different mechanisms may be used by cytosolic Bag1 and nuclear Bag1L for producing the same net effect on gross phenotypes, such as growth and survival. Future molecular comparisons of Bag1- and Bag1L-expressing cells, including use of cDNA arrays and proteomics methods, will likely provide additional insights into the molecular mechanisms responsible for the phenotypes conferred by Bag1 and Bag1L on breast cancer cells.

A curious difference in the correlation of cytosolic and nuclear Bag1 proteins with patient survival has been noted in retrospective analysis of some cohorts of breast cancer patients involving assessment of Bag1 expression by immunohistochemistry. Cytosolic Bag1 immunoreactivity was correlated with longer survival in women with early stage (stage I and II) breast cancer treated with lumpectomy and local radiation (11), whereas nuclear Bag1 immunostaining was associated with shorter survival among a diverse group of breast cancer patients (stages I–IV) treated heterogeneously (10). Although multiple technical differences in the methods used for detection of Bag1 proteins by immunohistochemistry and differences in the patient

cohorts analyzed may account for these divergent associations with clinical outcome (11, 30), it will be of interest to see Bag1 and Bag1L expression compared with patient survival in additional clinical correlative studies involving well-controlled cohorts of patients treated uniformly.

The xenograft analysis of ZR-75-1 cells expressing Bag1, Bag1L, or  $\Delta$ C mutants of these proteins provides the first evidence that Bag1 proteins can regulate the growth of tumors formed by breast cancer cells *in vivo*. Previously, overexpression of cytosolic Bag1 in a gastric carcinoma cell line was shown to increase *i.p.* tumor burden in immunocompromised mice (31), suggesting that Bag1 can play a role in enhancing tumorigenicity under some circumstances. Our data thus extend information about *in vivo* effects of Bag1 to another type of cancer (breast cancer) and also provide information about the nuclear Bag1L protein for the first time. Moreover, the finding that mutants of either Bag1 or Bag1L lacking the Hsp70-binding domain greatly suppress tumor cell growth rates in mice suggests that both of these proteins may play important roles in the growth of at least some breast cancers. Interestingly, Bag1 $\Delta$ C and Bag1L $\Delta$ C suppressed both estrogen-dependent and estrogen-independent growth of ZR-75-1 cells *in vivo*. Consequently, Bag1 and Bag1L may represent targets for drug discovery or for other therapeutic strategies designed to oppose breast cancer.

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