**BAG-1 is a Novel Cytoplasmic Binding Partner of the Membrane Form of Heparin-binding EGF-like Growth Factor: A Unique Role for proHB-EGF in Cell Survival Regulation**

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Abbreviations

EGFR: epidermal growth factor receptor
HB-EGF: heparin-binding EGF-like growth factor
hsp70/hsc70: 70 kDa heat shock protein/heat shock chaperone
TGFα: transforming growth factor-α
GST: glutathione-S-transferase
H₂O₂: hydroxyl peroxide
AP: alkaline phosphatase
PAGE: polyacrylamide gel electrophoresis
CHO: Chinese hamster ovary
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
PBS: phosphate buffer saline
BSA: bovine serum albumin
Abstract

Several cell functions related to growth and survival regulation have been attributed specifically to the membrane form of heparin-binding EGF-like growth factor (proHB-EGF), rather than to the diffusible, processed HB-EGF isoform. These findings suggest the existence of a functional binding partner specifically for the membrane form of the growth factor. In this study we have identified the prosurvival cochaperone, BAG-1, as a protein that interacts with the cytoplasmic tail domain of proHB-EGF. Interaction between BAG-1 and the 24-amino acid proHB-EGF cytoplasmic tail was initially identified in a yeast two-hybrid screen and was confirmed in mammalian cells. The proHB-EGF tail bound BAG-1 in an hsp70-independent manner and within a 97 amino acid segment that includes the ubiquitin homology domain in BAG-1 but does not include the hsp70 binding site. Effects of BAG-1 and proHB-EGF co-expression were demonstrated in cell adhesion and cell survival assays and in quantitative assays of regulated secretion of soluble HB-EGF. Because the BAG-1 binding site is not present on the mature, diffusible form of the growth factor, these findings suggest a new mechanism by which proHB-EGF, in isolation from the diffusible form, can mediate cell signaling events. In addition, because effects of BAG-1 on regulated secretion of soluble HB-EGF were also identified, this interaction has the potential to alter the signaling capabilities of both the membrane-anchored and the diffusible forms of the growth factor.
Introduction

Soluble ligands for the ErbB family of receptor tyrosine kinases, the EGF-like growth factors, are initially expressed as membrane-anchored precursors, which undergo regulated proteolysis to release the mature mitogens into the extracellular space. An interesting property of the membrane-anchored forms of the EGF-like factors is that they can be biologically active while still tethered to the membrane (1-3). This property has been termed juxtacrine signaling, as distinct from autocrine or paracrine signaling, which requires processed forms of the growth factors to diffuse from the cell and mediate receptor activation.

Heparin-binding EGF-like growth factor (HB-EGF) is a direct activating ligand for the EGF receptor (EGFR/ErbB1) and the related tyrosine kinase, ErbB4 (reviewed in (4)). HB-EGF gene expression and protein synthesis are upregulated in response to cell stress, consistent with a cytoprotective function for the molecule. The secreted form of HB-EGF is proteolytically processed from a membrane-anchored precursor, proHB-EGF, expressed by many epithelial, fibromuscular and other cell types. Membrane proHB-EGF has been demonstrated to exhibit a variety of biological activities, including stimulation of DNA synthesis, enhanced intercellular adhesion (2), regulation of cell survival (5,6), and binding and internalization of diphtheria toxin (7). Some of these activities are known to result from proHB-EGF binding to its cognate receptor on adjacent cells or with accessory proteins resident in the cell membrane (3,8). Several reports have provided evidence that proHB-EGF-dependent signaling cannot always be replicated by the soluble forms of the molecule (5,9,10), suggesting that the membrane-bound forms are involved in signaling events distinct from those mediated by the diffusible forms.
In this study we report that the cytoplasmic tail of proHB-EGF interacts with BAG-1, a multifunctional protein first identified as a binding partner of the anti-apoptotic protein Bcl-2 (11). BAG-1 associates with several signaling molecules and is capable of suppressing apoptosis. Our findings suggest a novel mechanism through which proHB-EGF might mediate physiological processes related to growth, adhesion and cell survival.
Materials and Methods

Yeast two hybrid analysis. LexA-based yeast two-hybrid screening was performed as described (12), using a constitutively expressed proHB-EGF-tail bait fusion and a galactose inducible prey-fusion library. A DNA fragment encoding the intracellular domain of human HB-EGF (amino acids 185-208) was generated by polymerase chain reaction (PCR) using the primer pairs: 5’-CTCGAATTCAGGTACCATAGGAGAGGAGGT-3’ and 5’-TCTCTCGAGGTGGGAATTAGTCATGCCCAA-3’. The fragment was subcloned into the EcoRI and XhoI sites of the vector pEG202, such that the HB-EGF cytoplasmic tail fragment was in frame with the LexA DNA binding domain. Intrinsic transcription activation activity of the bait plasmid pLexA-HB-EGF-tail was negligible. The cDNA library used for screening, a gift from Dr. Russell Finley, Jr, was generated from poly(A)+ RNA isolated from a human prostate carcinoma (LNCaP) xenograft grown in an athymic mouse host. Potential interactors were screened by auxotrophic selection on plates supplemented with galactose or glucose, but lacking histidine, uracil, tryptophan and leucine (Gal/-HUTL or Glu/-HUTL), and for the ability to metabolize X-Gal on Gal/X-gal/-HUT or Glu/X-gal/-HUT plates. Positive colonies grew on Gal/-HUTL plates and also appeared blue on Gal/X-gal/-HUT plates.

GST-fusion protein construction and pull-down assay. The GST-HB-EGF tail fusion construct was generated by ligating an EcoRI-XhoI HB-EGF tail PCR product into pGEX-4T1 (Pharmacia). The GST-BAG-1 fusion construct was generated by excising a DNA fragment encoding the 219 amino acid form of mouse BAG-1 from the prey plasmid pJG45-mBAG-1 (clone#B11), using EcoRI and XhoI, and cloned into pGEX-4T1. N- and C-terminally truncated variants of the GST-BAG-1 fusion construct were generated by PCR from pJG45-mBAG-1 using the following primers: GST-BAG1 (∆C): 5’-CAG ACC GAA TTC ATG GCC...
AAG ACC G-3' and 5'- TCC AGA ATT CGA GGT GTT-3' and 5'- GAC AAG CCG ACA ACC TTG ATT GGA G-3'. PCR was performed using a high fidelity Taq polymerase (Gibco/BRL, Gaithersburg, MD) and products were subcloned into pGEX-4T1 and sequenced. All GST fusion proteins were purified as described (11).

The GST fusion proteins pull-down assay was employed as described (11). BAG-1-enriched lysates were generated from COS7 cells overexpressing recombinant BAG-1, or from the human prostate carcinoma cell lines, LNCaP and PC-3, in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Roche, Indianapolis, IN). HB-EGF-enriched lysates from transfected cells were prepared in 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40 supplemented with protease inhibitor cocktail. Cell lysates (~500 µg total protein) were incubated with 5µg GST fusion proteins. BAG-1 was detected with the following antibodies: anti-human BAG-1 (Oncogene Research Products, Cambridge, MA), anti-BAG-1/RAP46/HAP1 (Neomarkers, Union City, CA) and/or anti-BAG-1 (clone 4A2, MBL, Nagoya, Japan). Anti-alkaline phosphatase (AP) antibody (Ab) (anti-human placental AP) was from Zymed (San Francisco, CA) and anti-hsp70/hsc70 was from Santa Cruz Biotechnology (Santa Cruz, CA).

Far-Western blot analysis. Far-Western blotting was modified from the filter binding assay as described (11). Briefly, 1 µg each GST and GST-HB-EGF\textsubscript{185-208} were size-fractionated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membrane. Membranes were preblocked by 20 mM HEPES (pH 7.4), 75 mM KCl, 2.5 mM MgCl\textsubscript{2}, 2 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Triton X-100 containing 3% BSA for 1 h and then incubated overnight at 4°C in the same solution containing 1 mM AEBSF, 3% BSA, 1% (v/v) lysate from pcDNA3.1/BAG-1 transfected COS7 cells. Bound BAG-1 on the
membrane was detected with anti-BAG-1 monoclonal Ab (MBL) immunoblotting and visualized by ECL (Chemicon, Temecula, CA).

**Cell culture and transfection.** LNCaP and PC-3 cells were grown in RPMI-1640/10% FBS. CHO-K1 cells were cultured in F12K/10%FBS. NRK52E and COS7 cells were grown in DMEM/10% FBS. MC2 cells were cultured in T medium as described (13). All cells were maintained in a humidified atmosphere of 95% air/5% CO$_2$ at 37°C.

The proHB-EGF-AP fusion construct (pRc/CMV-proHB-EGF-AP) and the tail-less form of this construct (pRc/CMV-proHB-EGF (Δtail)-AP) have been described (13). A cDNA fragment encoding BAG-1 was excised from pJG45-mBAG-1 (clone #B11) and subcloned into the EcoRI and XhoI sites of either pcDNA3.1/Myc-His (+) or pcDNA6/His [Invitrogen, Carlsbad, CA] using standard protocols. NRK52E cells expressing proHB-EGF or the tail-less form of proHB-EGF were created by transfecting cells with the plasmids described above using FuGENE6 transfection reagent (Roche). Double-stable (HB-EGF+BAG-1) CHO transfectants were generated as in (10). Initially cells were transfected with pRc/CMV-proHB-EGF-AP or with the empty vector and selected in G418-containing medium. Each population was then transfected with either pcDNA6/His-BAG-1 or the control vector, pcDNA6/His-LacZ and stable transfectants were selected by blasticidin (Invitrogen). MC2-proHB-EGF-AP and MC2-proHB-EGF(Δtail)-AP cloned cells have been described (13) and were double transfected by pcDNA6/His-BAG-1 or pcDNA6/His-LacZ as above. Cell populations were selected and maintained in medium supplemented with 300 µg/ml G418 and/or 5 µg/ml blasticidin.

**MTT and DNA fragmentation assays** were performed as described (14,15).
**Immunofluorescence Confocal Microscopy.** BAG-1-transfected MC2-proHB-EGF-AP cells were either treated or untreated by 0.4 mM etoposide for 24 h and cells were fixed with incubation with 2% paraformaldehyde for 1 h at room temperature. Cell permeabilization was performed with 0.1% Triton-X-100 in PBS for 3 min on ice. Cells were washed with PBS, blocked for 30 min with PBS/0.1% BSA/0.075% glycine (blocking buffer) and incubated with anti-AP monoclonal Ab (8B6, Sigma) (1:250) and rabbit polyclonal anti-BAG-1 antibody (1:1000) (N20, Santa Cruz) diluted in blocking buffer for 1 h at room temperature. After washing with blocking buffer, cells were incubated with Texas-red-conjugated donkey anti-mouse IgG and fluorescein (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, Inc. West Grove, PA) for 45 min. Slides were washed extensively with blocking buffer prior to mounting and were viewed using a BioRad 1024 Laser Scanning Confocal Imaging System. Up to forty serial optical sections (approximately 0.5 µm section thickness) were collected on informative cells. Individual channels of double labeled cells were collected as two separate series and merged in Confocal Assistant (written by Todd Breljie).

**HB-EGF secretion assay.** Secretion of HB-EGF was measured by determining levels of alkaline phosphatase (AP) in the medium using cells expressing proHB-EGF-AP fusion proteins as described previously (13). Briefly, 40,000 cells/well were seeded in 24-well plates and 24 h later, cells were stimulated by etoposide at the different concentrations under serum free conditions. Medium was collected 24 h later and AP activity was measured spectrophotometrically.

**Statistical Analysis.** Data were compared using a paired Student t-test. P values less than 0.05 were considered significant.
Results

The HB-EGF tail domain interacts with the prosurvival protein, BAG-1

The 24-residue cytoplasmic tail of proHB-EGF exhibits a high degree of inter-species sequence conservation (95% amino acid identity between mouse and human), suggesting an important functional role for this region of the protein. In a previous study, proHB-EGF was shown to protect NRK52E renal epithelial cells from apoptosis induced by H$_2$O$_2$ or etoposide treatment (9). Soluble HB-EGF was not able to replicate this cytoprotective effect. In order to determine if the proHB-EGF tail is involved in this process, NRK52E cells were transfected with intact proHB-EGF or proHB-EGF tail-deleted expression constructs. Cells expressing the two forms of the protein were then challenged with etoposide or H$_2$O$_2$. NRK52E cells expressing the proHB-EGF construct exhibited less apoptosis than vector-only control cells (Fig. 1), consistent with findings reported by Takemura et al. (9). In contrast, cells expressing the tail-deleted construct exhibited a similar level of apoptosis to the vector-only cells, suggesting a role for the tail domain in cytoprotection from apoptosis inducers.

These observations led us to search for proteins that interact with the proHB-EGF cytoplasmic domain. We screened a yeast two-hybrid expression library, constructed from a human prostate (LNCaP) xenograft tumor growing in a mouse host, with the proHB-EGF tail domain (a.a. 185-208), using a LexA-based system. Approximately $10^6$ independent clones were screened. From the 6 strongest potential interactors identified in the screen, two clones, pJG45-B11 and pJG45-E68 (Fig. 2A), each contained the entire open reading frame of the short form of the mouse protein, BAG-1 (219 amino acids, expected MW 24.5 kDa) (11). These two clones are identical but were derived from two
independent yeast transformations. In follow-up experiments, BAG-1 and the HB-EGF tail also interacted in the yeast two-hybrid system when the cDNAs were switched into the opposite (prey<-->bait) plasmid vectors.

To confirm the association between BAG-1 and HB-EGF, a GST fusion protein containing the HB-EGF cytoplasmic tail (GST-HB-EGF\textsubscript{(185-208)}) was constructed and used in pull-down assays. COS7 cells were transiently transfected with expression plasmids encoding mouse BAG-1. Lysates from these cells or from LNCaP cells (to test for binding with the native/endogenous, human form of BAG-1) were incubated with purified GST-HB-EGF\textsubscript{(185-208)}. A complex was formed between GST-HB-EGF\textsubscript{(185-208)} and BAG-1 but not between BAG-1 and GST alone (Fig. 2B). The converse experiment was also performed with a GST-BAG-1 fusion protein, using lysates from cells expressing AP-tagged proHB-EGF. In these experiments, a complex was formed between proHB-EGF and GST-BAG-1, but not between proHB-EGF and GST, or between GST-BAG-1 and proHB-EGF in which the tail domain was deleted (Fig. 2C). Complex formation between BAG-1 and the proHB-EGF tail was also demonstrated by Far-Western blot, in which the HB-EGF tail was immobilized and the interaction occurred on blotting membranes instead of in solution (Fig. 2D).

The 219 amino acid form of BAG-1 identified in the screen contains a ubiquitin homology domain (residues 37-73) and a central region (residues 90-172) that binds to Bcl-2 (11). Its carboxyl-terminal domain is required for direct interaction with the ATPase domain of hsp70 heat shock protein (16). We generated a GST-BAG-1(\Delta C) construct (residues 1-97), containing the ubiquitin homology region, and GST-BAG-1 (\Delta N) (residues 100-219), which carries binding sites for most of the known BAG-1 interactors. Complex formation with proHB-EGF was observed with GST-BAG-1 and GST-BAG-1(\Delta C), but not with GST-BAG-1(\Delta N). Complex formation did occur, however, between GST-BAG-
1(ΔN) and hsp70 (Fig. 2E), demonstrating the capability of GST-BAG-1(ΔN) to bind to a known BAG-1 binding protein despite its failure to bind to proHB-EGF. This result also indicates that the BAG-1 interaction with proHB-EGF is not mediated by hsp70 and it rules out the possibility that aberrant folding of GST-BAG-1(ΔN) is the reason for the absence of binding to HB-EGF. In a reciprocal experiment, GST-HB-EGF (185-208) was also able to form a complex with endogenous BAG-1 (MW 33-35 kDa) and endogenous hsp70 from human (LNCaP) cells (Fig. 2F).

We also investigated the dynamics of the BAG-1/HB-EGF tail interaction in cells induced to undergo apoptosis. LNCaP cells were treated with wortmannin, a PI3-kinase inhibitor that rapidly induces apoptosis in this cell line (15), and lysates were used in pull-down experiments. Interestingly, complex formation between the HB-EGF tail and endogenous BAG-1 diminished in a time-dependent manner following wortmannin treatment (Fig. 2G). Similar results were obtained when wortmannin-insensitive PC-3 cells were induced to undergo apoptosis by treatment with staurosporine, a protein kinase inhibitor (data not shown). These results suggest that the BAG-1/proHB-EGF interaction is not favored when cells undergo programmed cell death.

Taken together, these experiments 1) demonstrated a direct interaction between BAG-1 and the proHB-EGF cytoplasmic domain, 2) localized the HB-EGF interacting domain to within residues 1-97 of BAG-1 and 3) also revealed that BAG-1 can form a ternary complex with both proHB-EGF and hsp70 through interactions with these proteins at distinct binding sites.
To explore the possibility of a functional interaction between proHB-EGF and BAG-1, CHO cell populations were engineered sequentially to stably express either BAG-1, proHB-EGF, or both proteins. BAG-1+proHB-EGF-expressing cells exhibited a more epithelial-like cellular morphology, in comparison to cells expressing either BAG-1 or proHB-EGF alone or control vectors, or the parent cell, all of which exhibited a more fibroblastic appearance (Fig. 3). These data suggest that coexpression of both proteins confers functional properties on transfected cells that are not seen when each protein is expressed separately.

A similar requirement for proHB-EGF and BAG-1 co-expression to change a cellular phenotype was observed in other assays. BAG-1+proHB-EGF-expressing cells exhibited quantitatively reduced cell adhesion, as measured by sensitivity to trypsin/EDTA treatment, in comparison to cells expressing either BAG-1 or proHB-EGF or control plasmids (Fig. 4A). The presence of BAG-1 with proHB-EGF in CHO cell transformants also affected the sensitivity of these cells to certain apoptotic stimuli. BAG-1+proHB-EGF cells demonstrated increased resistance to apoptosis induced by etoposide, a topoisomerase inhibitor, in comparison to cells expressing either BAG-1 or proHB-EGF alone (Fig.4B). This resistance to apoptosis induction appeared to be confined to specific survival pathways, however, because BAG-1+proHB-EGF cells did not show synergistic protective effects when apoptosis was induced by staurosporine (data not shown).

We also compared secretion of soluble HB-EGF in response to apoptotic stimuli in proHB-EGF- and proHB-EGF(Δtail)-expressing MC2 cells transfected with BAG-1 and vector only. Etoposide treatment induced rapid secretion of soluble HB-EGF in cells expressing transfected BAG-1 but not in cells transfected with an empty vector (Fig. 5A). In contrast, in cells expressing a tail-deleted form of proHB-EGF, transfection with BAG-1 did not alter the secretion response to
etoposide. EGFR levels in proHB-EGF and proHB-EGF(Δtail) expressing cells were equivalent (Fig. 5B), indicating that differential capture of the soluble HB-EGF ligand by the EGFR cannot account for the observed differences in the secretion response. Similar results were observed in the CHO cell background (data not shown). These data suggest that the proHB-EGF tail is involved in regulated processing of the cell-associated form of the growth factor to the soluble form and that BAG-1 is capable of modulating this process in a manner that is dependent on the presence of the tail domain.

Immunofluorescence confocal microscopy indicated that proHB-EGF and BAG-1 co-localized within cytoplasm vesicles and at the plasma membrane, consistent with the possibility that BAG-1 can affect trafficking and maturation of soluble HB-EGF (Fig. 6). BAG-1 and proHB-EGF colocalized sites diminished in frequency when cells were treated with etoposide.
Discussion

The results of this study demonstrate that the membrane form of HB-EGF interacts with the anti-apoptotic protein, BAG-1, and that this interaction is likely to have functional significance. The BAG-1/HB-EGF tail interaction was demonstrated in a number of independent assays, including yeast two-hybrid, GST-pull-down, and Far-Western blot methods. Evidence for colocalization of BAG-1 and proHB-EGF was also obtained with immunofluorescence confocal microscopy. In addition, cooperative effects of BAG-1 and proHB-EGF expression were observed in assays of cell adhesion, apoptosis, and growth factor secretion. Consistent with these findings, we found that deletion of the proHB-EGF tail diminished the growth factor’s cytoprotective function and resulted in the loss of the ability of etoposide to induce HB-EGF secretion, results that point to an important role for the tail region. The diverse effects of proHB-EGF and BAG-1 coexpression indicate that the BAG-1/HB-EGF interaction may impinge on a number of discrete signaling pathways. Further, because we were able to demonstrate effects of BAG-1 expression on secretion of soluble HB-EGF, this interaction may regulate physiological activities of both the processed as well as the membrane-anchored forms of the growth factor.

Importantly, our results suggest a novel mechanism whereby the membrane-anchored form of HB-EGF might alter cell function independently of the soluble form of the molecule. BAG-1 was originally identified as a Bcl-2-binding protein but is now known to interact with and regulate a number of signaling proteins. Because the proHB-EGF tail and membrane-anchoring domains are removed from the mature growth factor by proteolytic cleavage, these results provide the first unambiguous mechanism whereby the HB-EGF pro-form could mediate processes distinct from those conferred by processed HB-EGF.
Several previous studies have identified bioactivities solely attributable to proHB-EGF, although the reason for the distinction between the precursor and secreted forms is not clear because both proteins presumably function principally by activating identical high-affinity receptor tyrosine kinases. Interactions between proHB-EGF and several cell surface molecules have been identified previously, including interactions with CD9/DRAP27, a tetraspanin membrane protein, the $\alpha$3$\beta$1 integrin, and heparan-sulfated glycosaminoglycans, by mechanisms that are dependent on the proHB-EGF ectodomain (3,8,17). However, BAG-1 is the first protein to be identified that interacts with the proHB-EGF cytoplasmic domain in an ectodomain-independent manner. Because CHO cells express low or negligible EGFR levels (18), our observations also suggest the interesting possibility--although this was not formally tested in the present study--that the cooperative effects we observed between proHB-EGF and BAG-1 do not require the EGFR. Receptor-independent signaling by proHB-EGF may be related to cell-cell adhesive functions previously noted for this growth factor (2,8).

BAG-1 is an important regulatory protein that has been shown to have a variety of binding partners and a range of bioactivities, including protection from apoptotic signals (11,19), enhancement of cell motility (20) and regulation of transcription (21,22). BAG-1 has been reported to bind the cytoplasmic domain of the HGF receptor (14), the Raf-1 kinase (23) and several members of the steroid hormone receptor superfamily (21,24), and to be capable of altering the activity of these molecules. Multiple BAG-1 isoforms, with differing patterns of cellular localization, arise by alternate translation initiation. Human BAG-1L (MW~57 kDa), for example, is a nuclear as well as a cytosolic protein, while the “small” BAG-1 isoforms studied here (MW 29-36 kDa) are predominantly cytosolic (25). Like HB-EGF, the BAG-1 proteins appear to have an important function in stress-regulated signaling. Enforced BAG-1 expression can promote
cell survival independently of effects on cell proliferation, motility and invasive properties (19), suggesting that effects on cell survival signaling are distinct from mechanisms of cell growth or cell cycle transit. In a similar fashion to our results, antiapoptotic effects of BAG-1 can be enhanced by coexpression of BAG-1 binding proteins, such as Bcl-2 (11,26). From these and other data, a critical role for BAG-1 in cell signaling related to cell survival mechanisms, but also to other processes, can be inferred despite uncertainty as to its precise mechanism of action.

The heat shock protein, hsp70, has been reported to be a favored BAG-1 interactor. In this case, BAG-1 appears to function as a regulator of protein folding and/or trafficking by acting as a competitive antagonist of the cochaperone, Hip (27). Although a number of proteins are thought to associate with BAG-1 because of its ability to bind hsp70 (16), we demonstrate in this study that proHB-EGF binds to a C-terminal deletion mutant of BAG-1 which does not bind hsp70 and that the HB-EGF interaction site on BAG-1 is distinct from the hsp70 interaction site. Furthermore, the HB-EGF tail-BAG-1 interaction occurs in yeast and yeast hsp70 is not a BAG-1 binding partner (16). Taken together, these data indicate that proHB-EGF and BAG-1 interact directly and in an hsp70-independent manner. This finding suggests that interaction of BAG-1 with proHB-EGF, and possibly with other regulatory proteins, may be functionally distinct from its role as a cochaperone in mechanisms of protein folding and/or stabilization.

BAG-1 is one of only a handful of proteins demonstrated to interact with the tail domains of membrane-bound EGF-like growth factors. TACIP18/syntenin and αA1 syntrophin were recently identified as specific interactors with the tail domain of proTGFα (28). TACIP18/syntenin appears to be involved in intracellular trafficking of TGFα. LIM kinase 1 (LIMK1) was identified as an
interactor with the tail domain of neuregulins and to co-localize with neuregulins at the neuromuscular synapse (29). LIMK1 does not interact with the proTGFα tail, suggesting that the cytoplasmic partners of membrane EGF-like growth factors are likely to play specialized roles. This hypothesis is consistent with the fact that ErbB1 ligands, despite similar receptor binding affinity to their primary receptor(s), do not show identical patterns of receptor transactivation or similar patterns of intracellular localization of their membrane forms and thus are functionally specialized.

In conclusion, our findings provide evidence that the prosurvival protein, BAG-1, is a functional binding partner of the membrane form of the receptor tyrosine kinase ligand, HB-EGF. This interaction could credibly alter aspects of cell signaling relating to unique functions of both the membrane as well as the soluble HB-EGF isoforms.
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Figure Legends

Figure 1. Deletion of the proHB-EGF cytoplasmic tail abolishes the cytoprotective effects of proHB-EGF expression in NRK52E cells. Transfected cells were stimulated by 0.5 mM H$_2$O$_2$ or 44.5 µM etoposide for 24 h. DNA fragments released into the cytoplasm were collected for agarose gel electrophoresis and visualized by ethidium bromide staining.

Figure 2. Interaction between proHB-EGF and BAG-1. (A) BAG-1 is a binding partner for the proHB-EGF tail in yeast. Transformed yeast colony (clone# 11) was streaked onto medium lacking histidine, uracil, tryptophan, leucine, and containing galactose (Gal/-HUTL) or glucose (Glu/-HUTL) as carbon source and growth was monitored 3 d later. The LacZ reporter gene was also monitored in medium containing X-gal (Gal/X-gal/-HUT or Glu/X-gal/-HUT). (B) GST-HB-EGF$^{(185-208)}$ pull-down assay: GST fusion proteins were incubated with cell lysates and the binding proteins were co-precipitated with glutathione-agarose and subjected to SDS-PAGE followed by anti-BAG-1 Western blot. Cell lysates were from COS7 cells transfected with pcDNA3.1/Myc-BAG-1 (upper panel) or human LNCaP cell lines (lower panel). (C) GST-BAG-1 pull-down experiment with proHB-EGF-AP-enriched cell lysates followed by detection of proHB-EGF-AP fusion protein with anti-AP Ab. (D) Far-Western blot: GST-HB-EGF$^{(185-208)}$ fusion proteins (1 µg) were subjected to SDS-PAGE and transferred to Immobilon-P membrane (left panel). The membrane was incubated with cell lysates from COS7 cells overexpressing BAG-1, followed by anti-BAG-1 immunodetection (right panel). (E) Identification of the BAG-1 domain responsible for proHB-EGF binding. GST-BAG-1 pull-down assay followed by anti-AP Western blot as in C (top). The same membrane was stripped and re-probed with anti-hsp70/hsc70.
monoclonal Ab (middle). The structure of the GST-BAG-1 fusion proteins used are indicated in the diagram. (F) GST-HB-EGF\textsubscript{(185-208)} pull-down experiment with LNCaP cell extracts followed by anti-BAG-1 Western blot (upper panel). The membrane was stripped and re-probed with anti-hsp70/hsc70 monoclonal Ab (lower panel). (G) Changes in the association between proHB-EGF and BAG-1. LNCaP cells were treated with 100 nM wortmannin and cell lysates were collected at the indicated time points. The GST-HB-EGF\textsubscript{(185-208)} binding proteins were detected by anti-BAG-1 Ab (upper panel). Levels of BAG-1 in cell lysates are shown by Western blot in the lower panel.

**Figure 3.** Phase contrast micrographs of BAG-1 and proHB-EGF-expressing CHO cells. CHO cells were stably transfected with (A) empty vector + LacZ vector; (B) proHB-EGF + LacZ vector; (C) empty vector + BAG-1 and (D) proHB-EGF + BAG-1. Western blots of the cell lysates from the four cell populations (A to D) are shown in the lower panel.

**Figure 4.** Synergistic effects of proHB-EGF and BAG-1 expression in CHO cells. (A) Adhesion assay. Cell populations (50,000/well) were seeded in 24 well plates. 48 h later cells were treated by trypsin-EDTA (1:15 dilution) for the times indicated. The cells remaining on the plate were quantified by crystal violet staining. (B) Cell survival assay. The four transfected cell populations were seeded in 96 well plate (10,000/well) and 24 h later cells were treated with etoposide for 24 h. Cells were exposed to MTT for 4 h at 37\textdegree C prior to harvest. In comparison to the other 3 groups, *: P<0.0005, **: P<0.01.

**Figure 5.** Requirement of the proHB-EGF tail in BAG-1 regulated secretion of HB-EGF. (A) MC2/proHB-EGF and MC2/proHB-EGF(\textDelta\text{tail}) cells were transfected with a BAG-1 expression construct or empty vector. Stable double-transfected cell populations were stimulated with 0.22 mM etoposide for
24 h and HB-EGF levels in the conditioned medium were measured. *: P<0.0001.

(B) Western blot of the total cell lysates from the above MC2 cells stably transfected with (a) proHB-EGF + empty vector; (b) proHB-EGF + BAG-1; (c) proHB-EGF(Δtail) + empty vector; (d) proHB-EGF(Δtail) + BAG-1.

Figure 6. Co-localization of BAG-1 and proHB-EGF. Confocal immunofluorescence microscopic evaluation of proHB-EGF and BAG-1 in BAG-1 transfected MC2/proHB-EGF cells. Representative patterns of proHB-EGF (A and D) and BAG-1 (B and E) staining in unstimulated cells are shown. Representative examples of etoposide stimulated cells are shown in G and H. Merged images are shown in C, F and I. Etoposide untreated (-) and treated (+).
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Figure 1
Figure 2

A. Bait: HB-EGF tail
Prey: BAG-1

B. MW(kDa)

C. MW(kDa)

D. GST GST-HB-EGF (185-208)

E. GST fusion proteins

F. GST GST-HB-EGF (185-208)

G. Wortmannin (h)

Figure 2
Figure 3
Figure 4
Figure 5

Panel A: Bar graph showing HB-EGF secretion (% of control) for proHB-EGF and proHB-EGF (Δtail) with Vector only and BAG-1 conditions.

Panel B: Western blot analysis with bands labeled a, b, c, d for EGFR and Actin.
Figure 6
BAG-1 is a Novel Cytoplasmic Binding Partner of the Membrane Form of Heparin-binding EGF-like Growth Factor: A Unique Role for proHB-EGF in Cell Survival Regulation

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