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HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death

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The mechanisms by which apoptosis is prevented by survival factors are largely unknown. Using an interaction cloning approach, we identified a protein that binds to the intracellular domain of the hepatocyte growth factor (HGF) receptor. This protein was identified as BAG-1, a recently characterized Bcl-2 functional partner, which prolongs cell survival through unknown mechanisms. Overexpression of BAG-1 in liver progenitor cells enhances protection from apoptosis by HGF. Association of the receptor with BAG-1 occurs in intact cells, is mediated by the C-terminal region of BAG-1 and is independent from tyrosine phosphorylation of the receptor. Formation of the complex is increased rapidly following induction of apoptosis. BAG-1 also enhances platelet-derived growth factor (PDGF)-mediated protection from apoptosis and associates with the PDGF receptor. Microinjection or transient expression of BAG-1 deletion mutants shows that both the N- and the C-terminal domains are required for protection from apoptosis. The finding of a link between growth factor receptors and the anti-apoptotic machinery fills a gap in the understanding of the molecular events regulating programmed cell death.

Keywords: apoptosis/BAG-1/Bcl-2/HGF receptor/PDGF receptor

Introduction

Proliferation and cell death are considered two mechanistically related phenomena. According to this view, cells are programmed to suicide by default and require specific extracellular factors to survive (Raff, 1992; Raff *et al.*, 1993). Indeed, both the extracellular matrix and specific soluble cytokines have been shown to promote cell survival (Frisch and Francis, 1994; Harrington *et al.*, 1994). Nerve growth factor (NGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF)-1 are among the best characterized survival factors and prevent apoptosis by binding to the corresponding tyrosine kinase receptors. The molecular events linking tyrosine kinase receptors to the anti-apoptotic machinery of the cell have not been elucidated. A number of studies, however, suggest that, upon activation, these receptors promote cell survival by: (i) triggering specific signalling pathways; (ii) modulating the activity of anti-apoptotic molecules; and (iii) inhibiting cell death effectors (Snider, 1994; Steller, 1995).

Hepatocyte growth factor (HGF) is a multifunctional polypeptide which elicits mitogenic, motogenic and morphogenic activities in various cell types. HGF stimulates proliferation of epithelial and endothelial cells (Nakamura *et al.*, 1986; Bussolino *et al.*, 1992). HGF also triggers the 'scatter' effect by inducing cell dissociation and migration (Stoker *et al.*, 1987). Finally, HGF promotes formation of branching tubules by epithelial cells grown in collagen gel matrix (Montesano *et al.*, 1991; Brinkmann *et al.*, 1995; Medico *et al.*, 1996). Recently, it has been suggested that HGF acts as a survival factor for Rat pheocromocytoma cells (PC12) and has a role in promoting survival and differentiation of hepatocytes during development (Matsumoto *et al.*, 1995; Schmidt *et al.*, 1995). Moreover, HGF prevents cell death induced by inhibition of adhesion (Frisch and Francis, 1994). The receptor for HGF is the heterodimeric tyrosine kinase encoded by the c-*met* proto-oncogene (Giordano *et al.*, 1989a,b; Bottaro *et al.*, 1991; Naldini *et al.*, 1995). HGF receptor signalling is mediated by phosphorylation of a multifunctional docking site located in the C-terminal region which interacts with multiple SH2-containing signal transducers (Ponzetto *et al.*, 1994). The biological response is elicited by the concomitant activation of multiple intracellular effectors, including phosphatidylinositol 3-kinase and the *Ras* pathway (Graziani *et al.*, 1991, 1993; Ponzetto *et al.*, 1993).

We made use of an expression cloning approach to identify novel proteins capable of associating with the HGF receptor cytoplasmic domain. Using this technique, we identified BAG-1. This protein was originally cloned as a Bcl-2 binding molecule and represents the product of a novel type of cell death suppressor gene (Takayama *et al.*, 1995). BAG-1 can prolong cell survival through unknown mechanisms, and cooperates with Bcl-2 in the suppression of apoptosis (Takayama *et al.*, 1995). We found that increasing the level of BAG-1 by gene transfer enhances HGF- and PDGF-induced protection from apoptosis in liver progenitor cells. The interaction of BAG-1 with the receptor occurs independently from tyrosine phosphorylation of the receptor and is mediated by the C-terminal region of BAG-1. However, the full size protein is required for growth factor-mediated protection from apoptosis. Finally, we show that induction of apoptosis stimulates the association of BAG-1 with the receptors.

Results

Expression cloning of BAG-1 using the HGF receptor cytoplasmic domain

In order to identify novel molecules that associate with the HGF receptor, we made use of an expression cloning

approach. A mouse embryo cDNA library was screened using the recombinant HGF receptor cytoplasmic domain as a probe. Positive clones from an initial screening of 2×10^6 phages were identified based on their ability to bind the receptor. Upon sequencing, four independent isolates of different length (Cl-153, Cl-121, Cl-93, Cl-92) were found to contain the same cDNA and to match the sequence of the recently identified Bcl-2 binding protein BAG-1 (Figure 1a). This protein is the product of a novel type of cell death suppressor gene that prolongs cell survival through unknown mechanisms (Takayama *et al.*, 1995). All BAG-1 clones revealed open reading frames extending further 5′ with respect to the published sequence. However, no additional ATGs were found, and a single polypeptide with a mol. wt of ~29 kDa was detected upon stable or transient transfection in mammalian cells. The interaction between the proteins encoded by the BAG-1 clones and the cytoplasmic domain of the HGF receptor was proved further by an immobilized protein interaction assay (far Western blot). The cDNAs corresponding to the four BAG-1 clones were expressed in bacteria in-frame with the upstream vector sequence. Total extracts were blotted and hybridized with ^{32}P-labelled HGF receptor (Figure 1b). The immobilized BAG-1–GST fusion protein was also able to precipitate the baculovirus-expressed HGF receptor, showing that a specific interaction can take place in solution (Figure 1c). The BAG-1–receptor complex was not affected by high salt washes, indicating a tight association.

HGF-induced protection from apoptosis is enhanced by overexpression of BAG-1

Mouse embryo liver progenitor cells (MLP-29), expressing physiological levels of endogenous HGF receptor, were treated with staurosporin. This drug is a well-known apoptotic agent, and its effects can be counteracted by survival factors, such as insulin or PDGF, without a requirement for RNA or protein synthesis (Jacobson *et al.*, 1993, 1994; Takayama *et al.*, 1995). Micromolar concentrations of staurosporin induced apoptosis in MLP-29 cells as measured by pycnosis of the nuclei, trypan blue exclusion assay (Figure 2b and c) and loss of mitochondrial function (Figure 3). Addition of nanomolar concentrations of HGF partially suppressed these effects (Figure 2b and c). We then evaluated if BAG-1 could influence the observed anti-apoptotic action of HGF. To this end, vesicular stomatitis virus (VSV)-tagged BAG-1 was stably transfected in MLP-29 cells. Three independent clones, expressing different levels of BAG-1 (Figure 2a), were tested for sensitivity to staurosporin-induced apoptosis in the presence and in the absence of HGF. As expected, exogenous BAG-1 alone only slightly promoted cell survival (Figure 2b and d). However, the anti-apoptotic action of HGF was enhanced markedly in BAG-1 transfectants, and protection from cell death correlated directly with the levels of expression (Figure 2b). Moreover, in transfected cells, the anti-apoptotic activity of BAG-1 was dependent on the addition of exogenous HGF (Figure 2d). To assess whether overexpression of BAG-1 could counteract cell death induced by an apoptotic agent different from staurosporin, which is a known inhibitor of protein kinases, the DNA polymerization inhibitor etoposide was used. Identical results were obtained (Figure 3). Over-

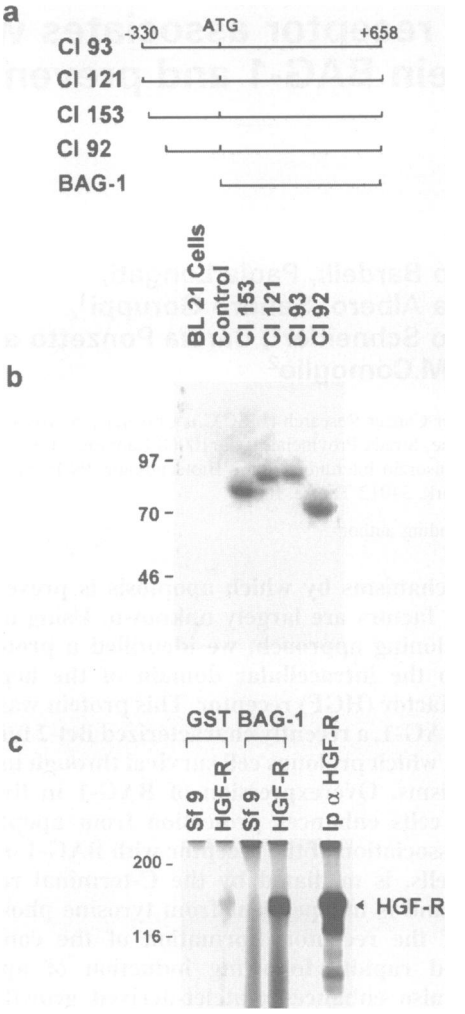

Fig. 1. Expression cloning of BAG-1 and characterization of the interaction with the HGF receptor. (**a**) A mouse embryo cDNA expression library was screened with the cytoplasmic domain of the HGF receptor. Four independent clones of different length (Cl-153, Cl-121, Cl-93, Cl-92) were found to contain the same cDNA and to match the sequence of the recently identified protein BAG-1. (**b**) Far Western blot showing the specific interaction between the isolated BAG-1 clones (Cl-153, -121, -93, -92) and the cytoplasmic domain of the HGF receptor. The cDNAs were expressed in bacteria in-frame with the upstream vector sequence; variations in size reflect the different 5′ lengths of the individual BAG-1 isolates. Total extracts were blotted and hybridized with ^{32}P-labelled HGF receptor. Controls included lysates from uninfected bacteria (BL-21) and from a clone expressing a non-related cDNA (control). (**c**) Characterization of the interaction between BAG-1 and the HGF receptor. Association of immobilized GST–BAG-1 with full size HGF receptor expressed in baculovirus. Lysates from uninfected (Sf9) or baculovirus-infected (HGF-R) cells were incubated with control (GST) or GST–BAG-1 beads, and bound HGF receptor was revealed by auto-phosphorylation. As a positive control, an aliquot of the lysate was immunoprecipitated with a specific antibody (ip-α-HGF-R). Data were confirmed by Western blot (not shown).

expression of BAG-1 *per se* slightly increased the viability of cells treated with staurosporin, but had no effect on cells treated with etoposide.

BAG-1 enhances PDGF-mediated protection from apoptosis and associates with the PDGF receptor

To assess whether overexpression of BAG-1 could influence the anti-apoptotic activity of growth factors other

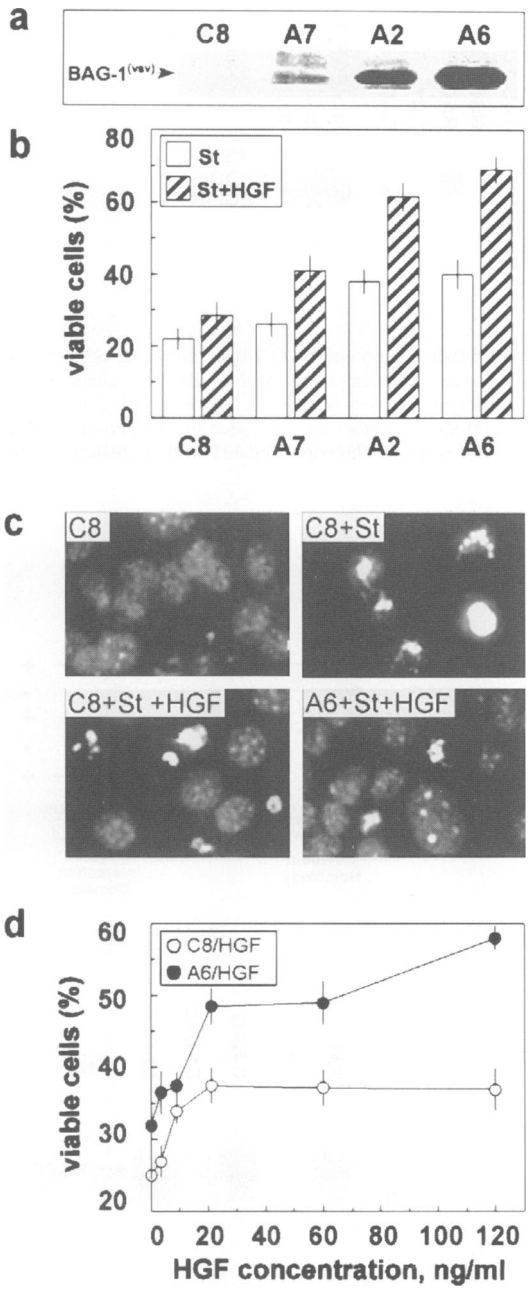

Fig. 2. BAG-1 overexpression enhances the anti-apoptotic action of HGF. (**a**) Western blot indicating the expression levels of transfected BAG-1–VSV in three different clones (A7, A2, A6) of liver progenitor cells (MLP-29). C8 indicates cells transfected with empty vector. (**b**) Anti-apoptotic effect of HGF in MLP-29 clones expressing different levels of BAG-1. Cells were incubated with staurosporin in the absence (open bars) or in the presence (dashed bars) of recombinant HGF (100 ng/ml). The percentage of viable cells was evaluated by trypan blue exclusion. Values (± SD) represent the mean of triplicate experiments. (**c**) MLP-29 cells transfected with empty vector (C8) or overexpressing BAG-1 (A6) were treated with staurosporin in the presence or absence of HGF, fixed and revealed with H32228 dye staining. (**d**) Control C8 cells (○) and BAG-1-overexpressing A6 cells (●) were incubated with staurosporin in the presence of HGF at the indicated concentrations. The percentage of viable cells was evaluated as above. Values (± SD) represent the mean of triplicate experiments.

than HGF, we treated MLP-29 with staurosporin and PDGF, epidermal growth factor (EGF) or serum. These cells express physiological levels of the corresponding

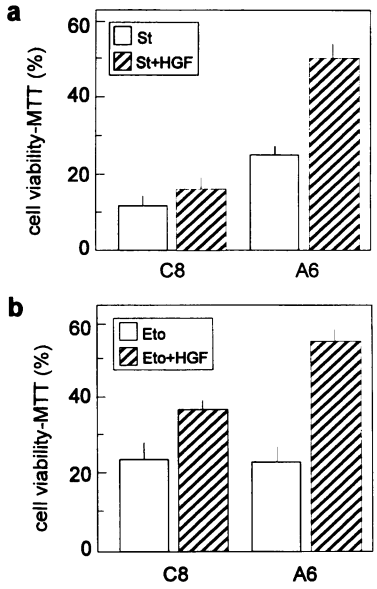

Fig. 3. BAG-1 and HGF protect from apoptosis induced by different agents. Control (C8) and BAG-1-transfected (A6) MLP-29 cells were treated with 0.1 mM staurosporin (**a**) or 40 mM etoposide (**b**) in the absence (open bars) or in the presence (dashed bars) of HGF for 16 h. Cell viability was evaluated as loss of mitochondrial functions according to the MTT assay.

receptors (Medico *et al.*, 1996). Figure 4a shows that BAG-1 enhanced the anti-apoptotic effect elicited by serum or PDGF. In this system, EGF had no protective effect. Next, we tested whether BAG-1 was able to form complexes with PDGF and EGF receptors. Lysates of Sf9 cells expressing recombinant baculovirus receptors were incubated with GST–BAG-1 immobilized on glutathione–Sepharose. Bound receptors were revealed by autophosphorylation in the presence of [^{32}P]ATP (Figure 4b) and immunoblotting (data not shown). In agreement with the biological response, we found that only the PDGF receptor associates with BAG-1 with high efficency.

Identification of the BAG-1 domain responsible for receptor binding

BAG-1 is a 29 kDa molecule whose N-terminal region shares homology with ubiquitin, and the C-terminal half is predicted to contain α-helices and amphipatic domains potentially involved in the formation of protein–protein complexes (Takayama *et al.*, 1995). To identify the domain responsible for receptor binding, BAG-1 deletion mutants were expressed as GST fusion proteins (Figure 5a) and tested for their ability to associate with the HGF receptor. The bound receptor was revealed by auto-phosphorylation. We found that the BAG-1 region of interaction with the receptor is restricted to the sequence included between amino acids 136–219 of the C-terminal domain (Figure 5b). Similar results were obtained using the PDGF receptor (Figure 5c).

Full-size BAG-1 is required for prevention of apoptosis by growth factors

To identify the BAG-1 functional domain(s) responsible for HGF and PDGF anti-apoptotic activity, N- and C-terminal deletion mutants were transiently over-expressed by microinjection in NIH 3T3 fibroblasts or

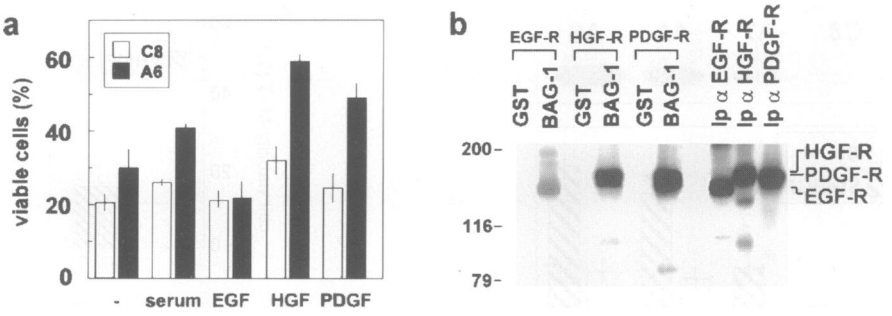

Fig. 4. Correlation between apoptosis prevention and BAG-1–receptor binding. (**a**) Control (white bars) and BAG-1-overexpressing cells (black bars) were incubated with staurosporin in the presence of serum (10%), EGF, HGF or PDGF at excess concentrations. Viable cells were quantitated as in Figure 2b. Values (± SD) represent the mean of triplicate experiments. (**b**) Binding of different receptors to BAG-1. Lysates of Sf9 cells expressing the indicated recombinant baculovirus receptor were incubated with immobilized BAG-1. Bound receptors were revealed by autophosphorylation. Immobilized GST was used as a negative control. As a positive control, aliquots of the lysates were immunoprecipitated with antibodies against the indicated receptors (Ip-α-EGF-R, Ip-α-PDGF-R, Ip-α-HGF-R).

transfected in liver progenitor cells. Microinjected cells were allowed to produce BAG-1 protein for 4 h. PDGF and staurosporin were then added and cells were incubated for a further 15 h. As observed in stable transfectants (Figure 2), transient overexpression of the full size BAG-1 sustained PDGF-mediated cell survival (Figure 6a). On the other hand, overexpression of a BAG-1 deletion mutant lacking the ability to bind the receptors (N-BAG) failed to sustain PDGF protection, abolishing the response in a 'dominant-negative' way. Interestingly, the expression of the deletion mutant which retains the ability to interact with the receptor (C-BAG) also failed to sustain PDGF anti-apoptotic activity (Figure 6a). Since NIH 3T3 cells do not express endogenous HGF receptor, liver progenitor cells were studied. Microinjection of these cells with different cDNAs resulted in an extremely low cell recovery. As an alternative to microinjection, BAG-1 mutants were overexpressed in these cells by transient transfection using lipofectin. Cells were allowed to produce BAG-1 proteins for 24 h, and apoptosis was induced by adding staurosporin in the presence or in the absence of recombinant HGF. As expected, wild-type BAG-1 sustained the HGF anti-apoptotic activity, whereas overexpression of either the N- or the C-terminal domain had no effect (Figure 6b). These results demonstrate that BAG-1-mediated protection from apoptosis, in response to HGF or PDGF, is regulated by a specific mechanism requiring a fully functional BAG-1 molecule.

HGF receptor–BAG-1 interaction occurs in intact cells and is independent from tyrosine phosphorylation

The C-terminal region of BAG-1 does not share homology with domains known to interact with tyrosine-phosphorylated receptors (Pawson, 1993; van der Geer *et al.*, 1995). We thus investigated whether binding requires HGF receptor autophosphorylation. Both BAG-1 and HGF receptor were co-expressed in COS-7 cells. VSV-tagged BAG-1 was co-transfected with either the wild-type receptor (Met^{wt}) or a kinase-defective HGF receptor mutant (Met^{lys-}). In both cases, a stable BAG-1–receptor complex was immunoprecipitated by antibodies directed against the HGF receptor, indicating that the interaction occurs *in vivo* and is not mediated by tyrosine phosphorylation

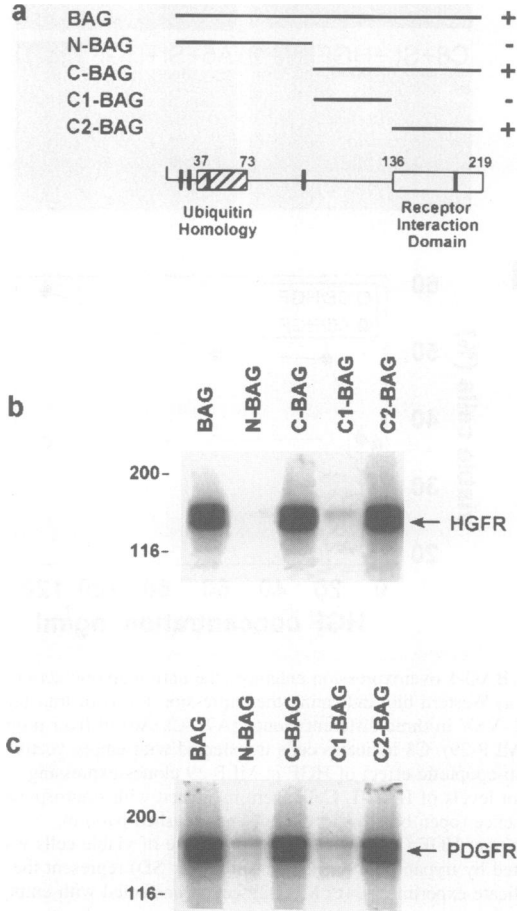

Fig. 5. Identification of the BAG-1 domain responsible for receptor binding. (**a**) Schematic representation of BAG-1 deletion mutants. N-BAG (aa 1–97); C-BAG (101–219); C1-BAG (101–135) and C2-BAG (136–219). The dashed box represents a region of homology with ubiquitin, the filled boxes glutamic acid-rich motifs, potentially involved in Ca^{2+} binding. The open box indicates the domain responsible for receptor binding (+). (**b** and **c**) Lysates from Sf9 cells expressing the HGF or the PDGF receptor were incubated with immobilized BAG-1 deletion mutants. Bound receptors were revealed by auto-phosphorylation. Full size BAG-1 was used as a positive control.

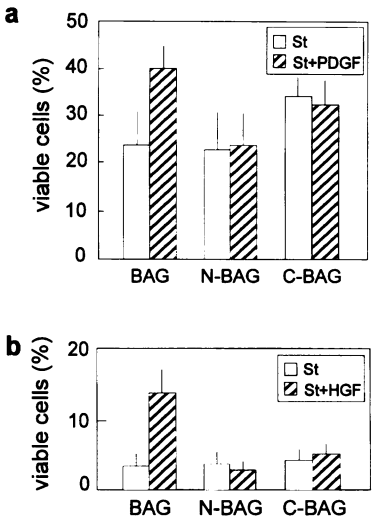

Fig. 6. Full size BAG-1 is required for prevention of apoptosis by growth factors. (**a**) NIH 3T3 cells were microinjected with VSV-tagged cDNAs of full size BAG-1 and deletion mutants. Cells were treated with staurosporin in the absence (open bars) or in the presence (dashed bars) of PDGF for 15 h (see Figure 1b). The percentage of viable cells (± SD) was evaluated by immunofluorescence analysis with anti-VSV antibodies. For each cDNA, 800 or more cells were microinjected in three independent experiments. (**b**) Liver progenitor cells (MLP-29) were transfected with VSV-tagged BAG-1 cDNAs. Cells were treated with staurosporin in the absence (open bars) or in the presence (dashed bars) of HGF for 15 h and the percentage of viable cells was evaluated by immunofluorescence analysis as above.

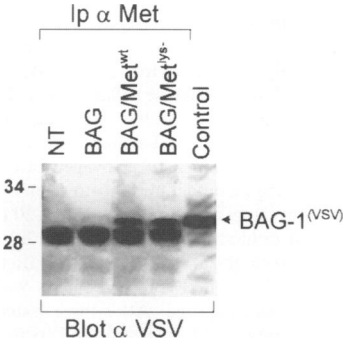

Fig. 7. Co-immunoprecipitation of BAG-1 and HGF receptor expressed in COS-7 cells. VSV-tagged BAG-1 was co-transfected with either a wild-type (Met^{wt}) or kinase-defective (Met^{lys-}) HGF receptor mutant. Cells were lysed, immunoprecipitated with antibodies against the HGF receptor and immunoblotted with anti-VSV antibodies. Negative controls included immunoprecipitates from mock-transfected cells (NT) or cell transfected with BAG-1 alone (BAG). The positive control is represented by total lysate from BAG-1-transfected cells (control).

(Figure 7). Moreover, real-time biosensor (Biacore™) analysis showed that the C-terminal receptor binding domain of BAG-1 does not interact with tyrosine-phosphorylated peptides derived from HGF receptor autophosphorylation sites (data not shown). These included the phosphopeptide corresponding to the double docking site, located in the receptor tail, which recruits multiple SH2-containing signal transducers and the phosphopeptides derived from the HGF receptor major phosphorylation site (Ponzetto *et al.*, 1993, 1994; Longati *et al.*, 1994). Finally, ligand stimulation does not influence BAG-1–HGF receptor association, nor is BAG-1 phosphorylated upon HGF or PDGF stimulation (data not shown).

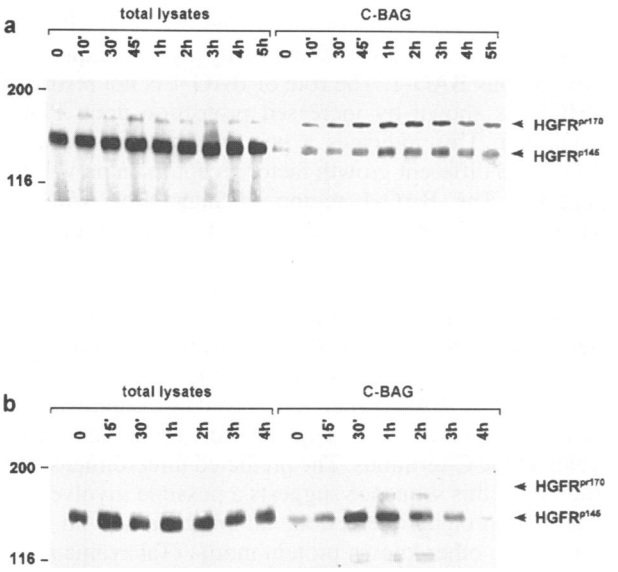

Fig. 8. Association of the HGF receptor with immobilized BAG-1 protein is increased upon induction of apoptosis. GTL-16 cells were incubated with staurosporin (**a**) or etoposide (**b**) for the indicated time, lysed and incubated with immobilized C-terminal BAG-1. Samples and total lysates were separated by SDS–PAGE and analysed by immunoblotting with anti-HGFR antibodies. The precursor ($HGFR^{pr170}$) and the mature HGFR β chain ($HGFR^{p145}$) are indicated.

HGF receptor–BAG-1 interaction is an early response to apoptotic agents

BAG-1–receptor interaction is not influenced by HGF binding or receptor phosphorylation. We therefore evaluated whether the association could be modulated in response to apoptotic agents. The human gastric carcinoma cell line (GTL-16) was used to perform association experiments. These cells express high levels of endogenous HGF receptor and proved to be a useful tool for monitoring receptor association with signalling molecules (Ponzetto *et al.*, 1994). Cells were treated with staurosporin or etoposide at various time intervals between 10 min and 5 h. Lysates were incubated with the immobilized BAG-1 C-terminal domain and the amount of associated HGF receptor evaluated. The association, already detectable at basal level in the absence of the drug, increased as early as 30 min after induction of apoptosis, reached the maximum between 1 and 2 h and decreased after 4 h. Densitometric analysis showed that treatment with staurosporin or etoposide increases the amount of HGF receptor bound to BAG-1 by 4- and 20-fold respectively.

Discussion

To identify novel molecules capable of interacting with the HGF receptor, we used its intracellular domain to screen an expression library. Four clones, independently isolated, contained the cDNA corresponding to the recently characterized anti-cell death gene BAG-1 (Takayama *et al.*, 1995). Originally identified as a Bcl-2 binding protein, BAG-1 represents a new type of anti-apoptotic molecule capable of prolonging cell survival and cooperating with Bcl-2 in the suppression of apoptosis. The identification of BAG-1 as an HGF receptor binding molecule prompted us to test the role of this molecule in the anti-apoptotic effects elicited *in vivo* by growth factors. In mouse liver

progenitor cells we observed that the effect of HGF on cell survival was increased markedly by overexpression of exogenous BAG-1. The role of BAG-1 is not restricted to HGF, as shown by increased protection upon PDGF stimulation. This indicates a possible interplay between BAG-1 and different growth factor receptors in preventing apoptosis. The BAG-1 region of interaction with the receptor has been mapped to the C-terminal domain. Although this domain binds HGF and PDGF receptors efficiently, overexpression of this region alone has no effects on the HGF and PDGF anti-apoptotic activities. These results indicate that BAG-1 is involved directly in HGF and PDGF survival signalling pathways.

The minimal BAG-1 region involved in receptor association corresponds to a sequence of 80 amino acids, located at the C-terminus. The predicted three-dimensional structure of this sequence suggests a possible involvement in protein–protein interaction, although it has no clear similarity to other known protein motifs (Takayama *et al.*, 1995). So far, two different modular domains (called SH2 and PTB) have been found to bind growth factor receptors, interacting with phosphotyrosine residues embedded in specific amino acid sequences (Songyang *et al.*, 1995). The BAG-1 region responsible for HGF receptor interaction has no homology either with the SH2 or with the PTB domain. Accordingly, the interaction of BAG-1 with the HGF receptor does not involve tyrosine phosphorylation. In fact BAG-1 was found to associate with a kinase-inactive receptor mutant, and formation of the complexes was not influenced by receptor auto-phosphorylation. Moreover, the C-terminal domain of BAG-1 did not interact *in vitro* with tyrosine-phosphorylated peptides derived either from the HGF receptor major phosphorylation sites, Y1234–Y1235, or from the multifunctional docking site for SH2 signal transducers Y1349–Y1356 (Ferracini *et al.*, 1991; Ponzetto *et al.*, 1994). Taken together, these data suggest that BAG-1–receptor interactions involve a novel mechanism, be it either direct or indirect. BAG-1 associates with the mature β chain of the receptor as well as with the receptor precursor. This finding suggests that the interaction occurs immediately after HGF receptor translation and that a pool of receptor molecules is constitutively associated with BAG-1. Interestingly, the number of receptors coupled to BAG-1 increases as an early response to apoptotic agents, suggesting that the formation of the complex is a regulated process. Regulation could be achieved either by a post-translational receptor modification (other than tyrosine phosphorylation) or by receptor coupling with an as yet unidentified BAG-1 adaptor molecule.

The detailed mechanism(s) through which the complexes between growth factor receptors and BAG-1 participate in protection from apoptosis remains to be elucidated. While the C-terminal region of BAG-1 interacts with growth factor receptors, the other half of the molecule, containing the ubiquitin-like domain, is available for other partners. Ubiquitin-like domains are present in a variety of molecules, including the mammalian Nedd8 and Gdx-1 genes and may be involved in protein degradation (Toniolo *et al.*, 1988; Takayama *et al.*, 1995). Alternatively, these domains may mediate protein–protein interactions (Toniolo *et al.*, 1988). In agreement with this hypothesis, a ubiquitin-like structure is present in the Raf-binding region of Ras and in the immunoglubulin binding domain of the streptococcal protein-G (Vijay-Kumar *et al.*, 1987; Nassar *et al.*, 1995). According to this view, BAG-1, with its ubiquitin-like domain, might act as a bridge linking the HGF and other growth factor receptors directly or indirectly to intracellular targets involved in control of apoptosis. In line with this hypothesis, we showed that a full size BAG-1 molecule is required to mediate the anti-apoptotic activity while either the receptor binding domain or the ubiquitin-like regions expressed separately are ineffective. BAG-1 protein might, thus, functionally act as an adaptor between tyrosine kinase receptors and the anti-apoptotic machinery of the cell.

Materials and methods

Reagents, antibodies and cell culture

All reagents used were from Fluka (FlukaChemie AG Buchs, Switzerland) and Sigma (Sigma Chemicals Co., St Louis, MO). Reagents for SDS–PAGE were from Bio-Rad (Biorad Laboratories, Cambridge, MA). Recombinant HGF was obtained from baculovirus-infected Sf9 cells (Naldini *et al.*, 1995). EGF and PDGF were from Sigma. Anti-VSV antibodies were purchased from Sigma and anti-HGF receptor monoclonal antibodies (Prat *et al.*, 1991) were obtained in this laboratory. Sf9, NIH 3T3, COS-7 and MDCK cells were from ATCC. GTL-16 and MLP-29 (mouse liver progenitor) cells were obtained as described (Giordano *et al.*, 1989a; Medico *et al.*, 1996). Cultures of mammalian cells were maintained in DMEM or RPMI supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2–air. Insect Sf9 cells were maintained in serum-free medium (SF900 Gibco) and infected with baculovirus as previously reported (Bardelli *et al.*, 1992).

cDNA library screening

The HGF receptor cytoplasmic domain was expressed as a GST fusion protein in Sf9 cells using the baculovirus system (Bardelli *et al.*, 1992) and purified on glutathione–Sepharose (Pharmacia). The receptor was labelled by autophosphorylation in the presence of [γ-^{32}P]ATP as described (Bardelli *et al.*, 1992) and eluted with free glutathione. A mouse embryo cDNA library in λEXlox (Novagen) was plated on BL21/DE3 cells according to the manufacturer's instructions. Filters were pre-blocked for 4 h at room temperature in Hyb buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, 0.1% Tween 20) containing 10% milk and hybridized overnight at 4°C with Hyb buffer containing 1% milk and 20 ng/ml of ^{32}P-labelled receptor. Filters were washed three times with Hyb buffer and exposed. After three rounds of screening, positives phages were converted to plasmids and sequenced.

Construction and transfection of tagged cDNAs

BAG-1 cDNA was subcloned in-frame with a C-terminal VSV tag (BAG–VSV) in pMT2SM-tag (Dr D.Shaap) and in pcDNA3 vectors (Stratagene). The BAG-1 cDNA was amplified by PCR using a sense primer which included the ATG start codon and an artificial *Eco*RI site at the 5′ end (5′-CAACATGGCCGAATTCGAGGAGATGGTCCAGACGGT-3′) (primer A) and an antisense primer containing an artificial *Not*I site (5′-CAGCCACTCTCCACGCGGCCGCCTTCAGCCAGGGCCAAGTTTGTAGAC-3′). The wild-type and kinase-defective (lys$^-$) HGF receptor constructs have been described previously (Ponzetto *et al.*, 1993; Zhen *et al.*, 1994). Transient expression in simian kidney COS-7 cells was performed using lipofectin (BRL) as previously reported (Ponzetto *et al.*, 1993). To obtain stable BAG-1 transfectants, MLP-29 cells were transfected with pcDNA3 containing BAG–VSV cDNA. After clonal selection with G418, BAG-1 expression was evaluated by immunoblotting with anti-VSV antibodies.

Western immunoblotting

After SDS–PAGE, proteins were transferred to Hybond-ECL membranes (Amersham) by high intensity wet blotting. Filters were probed with the appropriate antibodies and specific binding was detected by the enhanced chemiluminescence system (ECL, Amersham).

Preparation of bacterial constructs

GST fusion protein constructs were obtained by PCR amplification of *Bag-1* cDNA in Exlox using the thermal cycle *Taq*1 polymerase (Perkin

Elmer), followed by subcloning in pGEX-3X vector (Pharmacia) and expression in XL1-blue cells (Stratagene). For the GST–full size BAG construct, the amplification was performed using a sense oligonucleotide (primer A) encompassing the sequence which included the ATG start codon and an artificial *Eco*RI site at the 5′ end (5′-CAACATGGCCG-AATTCGAGGAGATGGTCCAGACGGT-3′) and an antisense oligonucleotide (primer B) corresponding to the sequence at the 3′ end of *Bag-1* cDNA and containing an artificial *Not*I site downstream of the stop codon (5′-CAGCCACTCTCCACGCGGCCGCCTTCAGCCAGG-GCCAAGTTTGTAGAC-3′). The amplified product was cleaved with *Eco*RI and *Not*I restriction enzymes and subcloned in pGEX-3X vector. For the other GST protein constructs, the same amplification and subcloning procedures were used with different couples of primers: for the GST–N-BAG construct, the primer A and an antisense primer containing an artificial *Sal*I site (5′-CTTCTTGTCGACTGCTCTTTT CACCAATTAACA-3′); for the GST–C-BAG construct, a sense primer (primer C) including an artificial *Eco*RI site (5′-CCAGAAGAAGAGGT TGAATTCAAGAAGCTGAAA-3′) and the primer B; for the GST–C1-BAG construct, the sense primer C and an antisense primer including an artificial *Not*I site (5′-TCAAGTTTGCAGAGAGCGGCCGCTTG-CAATT-3′); and for the GST–C2-BAG construct, a sense primer including an artificial *Eco*RI site (5′-TCTGGCTAAGGAATTCCAAGCGGAGG-CTCT-3′) and the antisense primer B.

Association experiments

The full size BAG-1 (amino acids 1–219), the N-terminal region (1–97), the C-terminal region (101–219) and the smaller portions C1 (101–135) and C2 (136–219) as GST fusion proteins were immobilized on glutathione–Sepharose and incubated with lysates of Sf9 cells infected with baculovirus containing the full-size HGF receptor cDNA (Bardelli *et al.*, 1992). After 0.5 M NaCl washing, bound receptors were labelled by autophosphorylation in the presence of [γ-^{32}P]ATP. In co-expression experiments, BAG-1–VSV cDNA and wild-type or lys$^-$ HGF receptor constructs were co-transfected in COS-7 cells. After lysis with EB buffer (100 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) containing protease inhibitors, immunoprecipitations with anti-HGF receptor antibodies were performed and samples analysed by SDS–PAGE and Western immunoblotting with anti-VSV antibodies. For the far Western blot procedure, the isolated cDNAs were expressed in BL21/DE cells in-frame with the upstream λEXlox vector sequence. After lysis, extracts were blotted and hybridized under the same conditions used for library screening.

Apoptosis assays

A total of 3×10^4 cells per flat-bottomed well were incubated with different concentrations of staurosporin (Sigma) or etoposide (Bristol-Myers Squibb) for the indicated times in the presence or absence of recombinant growth factors; the percentage of viable cells was evaluated by trypan blue exclusion assay (Jacobson *et al.*, 1994) or by MTT assay. In brief, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (Sigma) was added to cells (0.5 mg/ml) and, after 4 h of incubation at 37°C, the reaction was stopped by adding 10% SDS–0.01% HCl. Absorbance was measured at a wavelength of 595 nm. Alternatively, after treatment with staurosporin, cells were fixed with 3% paraformaldehyde and revealed with H32228 dye (Sigma) staining. When association experiments were performed upon apoptosis induction, GTL-16 cells were incubated with staurosporin or etoposide as indicated and lysed in EB buffer. Lysates were then incubated with the N- or C-terminal BAG-1–GST fusion protein (see above) immobilized on glutathione–Sepharose. After several washes, samples were separated by SDS–PAGE and analysed by immunoblotting with anti-HGF receptor antibodies.

Microinjection and transient transfection experiments

For the microinjection experiments, 1×10^4 NIH 3T3 cells/cm^2 were seeded on coverslips in 3 cm Petri dishes and used after 24 h. Microinjection was performed using an automated injection system (AIS; Zeiss, Oberkochen, Germany). Plasmid DNA dilutions in water were loaded into 1.2 mm diameter glass capillaries (Clark Electro Medical Instruments, Reading, UK) pulled using a capillary puller (Mecanex S.A., Geneva). NIH 3T3 cells were microinjected with VSV-tagged PMT2–BAG-1, PMT2–N-BAG or PMT2–C-BAG cDNAs (20 ng/μl). At 4 h after microinjection, 0.2 μM staurosporin was added to the medium in the absence or presence of PDGF (50 ng/ml) for 15 h. For transient transfection experiments, 3×10^4 MLP-29 cells/cm^2 were seeded on coverslips in 6 cm Petri dishes and transfected with VSV-tagged BAG-1 cDNAs (10 mg/dish). Cells were allowed to produce the protein for 36 h and then 0.1 μM staurosporin was added for 15 h, in the absence or presence of HGF (100 ng/ml). Microinjected or transfected cells were fixed, and immunofluorescence analysis was performed with a monoclonal anti-VSV (Sigma) antibody. The second antibody was a FITC-conjugated rabbit anti-mouse (Dako).

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Note added

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