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Mini-review

What's in the 'BAG'? – a functional domain analysis of the BAG-family proteins

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Abstract

Bcl-2-associated athanogene (BAG)-family proteins are BAG domain-containing proteins that interact with the heat shock proteins 70, both constitutive Hsc70 and inducible Hsp70. BAG-family proteins bind through the BAG domain to the ATPase domain of Hsc70/Hsp70. The BAG domain, approximately 110 amino acids in length, is a conserved region at the carboxyl terminus and consists of three anti-parallel α helices based on X-ray crystallography and NMR studies. The second and third α-helices of the BAG domain interact with the ATP-binding pocket of Hsc70/Hsp70. Currently, six human BAG proteins have been reported, four of which have been shown to functionally bind Hsc70/Hsp70. BAG-family proteins regulate chaperone protein activities through their interaction with Hsc70/Hsp70. Over-expression of BAG-family proteins is found in several cancers and has been demonstrated in the laboratory to enhance cell survival and proliferation. The anti-apoptotic activities of BAG-family proteins may be dependent on their interactions with Hsc70/Hsp70 and/or binding to Bcl-2. Both BAG-1 and BAG-3/CAIR-1 interact with Bcl-2 and have been shown to have a supra-additive anti-apoptotic effect with Bcl-2. Several N-terminal domains or motifs have been identified in BAG-family proteins as well. These domains enable BAG-family proteins to partner with other proteins and potentially alter the activity of those target proteins by recruiting Hsc70/Hsp70. BAG-family proteins participate in a wide variety of cellular processes including cell survival (stress response), proliferation, migration and apoptosis.

Keywords: Apoptosis; Stress response; Migration; Hsp70; Silencer of death domains; BcL-2; Survival; Proliferation; Chaperone

1. Introduction

Bcl-2-associated athanogene (BAG)-family proteins were originally identified by their ability to associate with the anti-apoptotic protein, Bcl-2 [1,2]. BAG-family proteins were also found to interact with heat shock proteins 70 (Hsc70/Hsp70) and can modulate, either positively or negatively, the functions of these chaperone proteins. Therefore, BAG-family proteins are characterized as co-chaperones [3,4]. Currently, six human BAG proteins have been reported. However, only four of them (BAG-1, -3, -4 and -6) have been confirmed in vivo and shown to interact with Hsc70/Hsp70. BAG-family proteins contain a single BAG domain, except for BAG-5 which has four BAG repeats (Fig. 1). The BAG domain is a conserved region located at the C-terminus of the BAG-family proteins that binds the ATPase domain of Hsc70/Hsp70 [3,4]. The BAG domain is evolutionarily conserved, and BAG domain containing proteins have been described and/or proven in a

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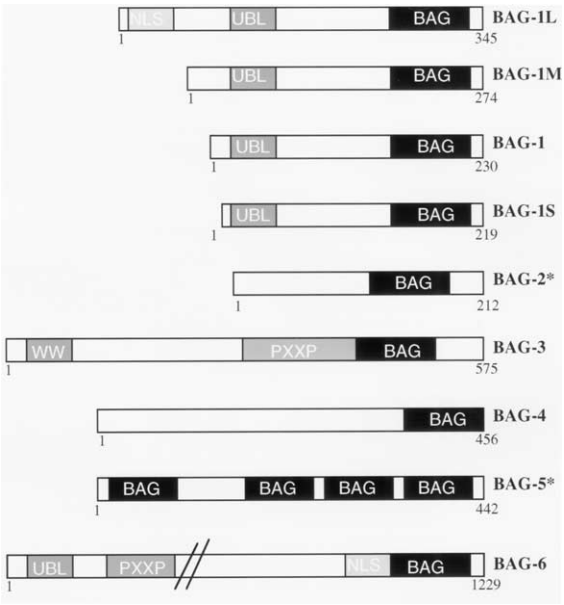

Fig. 1. Human BAG-family proteins. All six reported BAG proteins contain a BAG domain at their C-terminus. BAG-2 and BAG-5 proteins have not been confirmed in cells or tissues (*). Human BAG-family proteins contain other domains including nuclear localization signal (NLS), ubiquitin-like (UBL) domain, WW domain, and proline-rich regions (PXXP). Numbers below the linear peptide sequence indicate amino acid position in the proteins.

variety of organisms including mice, *Xenopus*, *Drosophila*, *Bombyx mori* (silk worm), *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Arabidopsis thaliana*. [4–8].

Human BAG-1, the founding member of this family, was initially discovered through a screen for Bcl-2 binding proteins. The BAG-1 gene encodes four isoforms of the BAG-1 proteins, expressed through alternative translation initiation sites (Table 1). BAG-1L (52 kDa) starts at a non-canonical CUG codon upstream of the AUG-driven mRNA transcript. Two in-frame downstream AUG codons give rise to BAG-1M (46 kDa) and BAG-1 (34 kDa), while BAG-1S (29 kDa) is believed to be generated by post-translational modification. BAG-1L is located primarily in the nucleus, while BAG-1M, -1, and -1S proteins are found mainly in the cytoplasm. BAG-1 and BAG-1M were occasionally found in the nucleus depending on the cell type and whether the cells were exposed to stress conditions [9,10].

BAG-3, a 74 kDa cytoplasmic protein, was identified by three different groups using three different approaches. BAG-3 was identified through a screen for ATPase domain-interacting proteins using a yeast two-hybrid system. The ATPase domain of Hsc70/Hsp70 was used as bait to pull down Hsc70/Hsp70 interacting proteins [4]. Bcl-2 interacting death suppressor (Bis) was discovered when a second group used a cDNA library to screen for Bcl-2 binding proteins [2]. In contrast, CAIR-1 (CAI-stress-1) was identified by subtraction hybridization between cells conditioned to grow in otherwise growth limiting concentrations of a calcium influx inhibitor, CAI [11].

BAG-4 was also identified through a yeast two-hybrid screen for proteins that interact with the ATPase domain of Hsc70/Hsp70 [4]. BAG-4 was also identified as silencer of death domains (SODD) [8]. It is a 60 kDa cytosolic protein that interacts with the death domain of tissue necrosis factor receptor-1 (TNF-R1) [12,13]. Recent data indicates that the TNF-R1 death domain contains an ATPase domain, and both Hsc70/Hsp70 and TNF-R1 interact with the BAG domain of BAG-4/SODD [14]. BAG-6, known as Scythe or HLA-B-associated transcript-3 (BAT-3), was initially purified from *Xenopus* egg extracts and

Table 1
Characteristics of proteins

BAG proteins	Alternative names	MW	Partner proteins	References
BAG-1S		29	Bcl-2, Hsc70/Hsp70, PDGF-, HGF-receptors	[3,4,8,53]
BAG-1		34	Bcl-2, Hsc70/Hsp70, Raf-1	[1,3,4,8,24]
BAG-1M	HAP46, RAP46	46	Bcl-2, Hsc70/Hsp70, retinoic acid receptor, glucocorticoid, estrogen, and thyroid hormone receptors	[1,3,4,8,9,52]
BAG-1L		52	Bcl-2, Hsc70/Hsp70, AR, VDR	[1,3,4,8,44–46]
BAG-3	CAIR-1, Bis	74	Bcl-2, Hsc70/Hsp70, PLC-γ	[2,4,11]
BAG-4	SODD	60	Hsc70/Hsp70, TNF-R1, DR-3	[8,12,13]
BAG-6	Scythe, BAT-3	150	Hsc70/Hsp70, *Reaper*	[5,15–17,23]

has been shown to interact in the nucleus with the *Drosophila* apoptosis-inducing factor, *reaper* [5,15]. hBAG-6, which resides in the nucleus, has been confirmed to interact with Hsc70/Hsp70 through the BAG domain [16,17]. BAG-family proteins may play roles in both the cytoplasm and the nucleus as indicated by their localization and their partner proteins (Table 1).

2. The BAG domain

The BAG domain of BAG-family proteins was first demonstrated to interact with Hsc70/Hsp70 using glutathione-S-transferase (GST) fusion proteins containing the BAG domain of BAG-1 [4]. The BAG domain was initially described as a conserved region of about 50 amino acids at the C-terminus of the BAG-1 protein [4]. However, recent studies using X-ray crystallography, multidimensional nuclear magnetic resonance (NMR), and limited proteolysis indicate that the BAG domain has 110–124 amino acids [7,18] and is comprised of three anti-parallel α helices, each approximately 30–40 amino acids in length (Fig. 2). The first and second helices (α1 and α2) interact with the serine/threonine kinase Raf-1 and the second and third helices (α2 and α3) are the sites of the BAG domain interaction with the ATPase domain of Hsc70/Hsp70 [8,18,19] and TNF-R1 [13]. Binding of the BAG domain to the ATPase domain is mediated by both electrostatic and hydrophobic interactions in BAG-1 and is energy requiring. Electrostatic interactions occur primarily through residues of Glu^{212}, Asp^{222}, Arg^{237} and Gln^{245}. Mutations at these sites of BAG-1 caused a substantial decrease in Hsc70 ATPase binding [18]. These four residues are highly conserved in all known BAG-family proteins (Fig. 2).

The BAG-1 BAG domain has been widely used for studying the interaction between the BAG domain and Hsc70/Hsp70. It binds to the ATPase domain of Hsc70/Hsp70 with a dissociation constant K_d of 1–3 μM [18]. BAG-1 was shown to stimulate the ATP hydrolysis of Hsc70 by accelerating the exchange of ADP/ATP, and thus acts as a nucleotide exchange factor for Hsc70/Hsp70 [20,21]. However, BAG proteins have also been found to negatively regulate the function of Hsc70/Hsp70. Both BAG-1 and BAG-6 have been shown to inhibit the chaperone-mediated refolding of denatured β-galactosidase and that this inhibitory effect is BAG-domain dependent [22,23]. Although further studies are required, the function of the BAG proteins as positive or negative regulators of Hsc70/Hsp70 may be related to the isoforms of BAG-1 and the timing of the BAG proteins involved in the chaperone mechanism.

3. BAG domain function

The BAG domain has been shown to contribute to the anti-apoptotic activity of BAG-family proteins. BAG-1 has been demonstrated to enhance cell resistance to apoptotic stimuli [1]. Although the detailed mechanism remains incompletely understood, some pathway dissection has been done. BAG-1 binds to

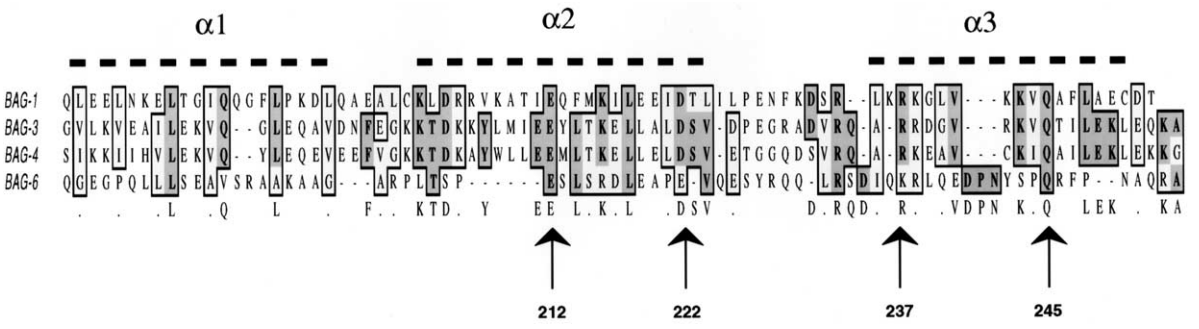

Fig. 2. The BAG-domain of human BAG-family proteins. The BAG domain consists of three anti-parallel helices (α1, α2 and α3). The α1 and α2 helices bind Raf-1 and the α2 and α3 helices bind Hsc70/Hsp70. Numbers below the peptide sequence indicate amino acid position of BAG-1 protein. Dark gray shading indicates identical residues, while light gray shading indicates similar residues.

the serine/threonine kinase Raf-1 or Hsc70/Hsp70 in a mutually exclusive interaction. BAG-1 promotes cell growth by binding to and stimulating Raf-1 activity [24]. The activated Raf-1 turns on its downstream extracellular signal-related kinase (ERK) activities and stimulates cell proliferation. When cells are under stress, such as heat shock, Hsp70 levels increase. The increased quantity of Hsp70 may compete with Raf-1 for BAG-1 binding. The binding of Hsp70 to BAG-1 diminishes Raf-1 signaling and inhibits subsequent events, such as DNA synthesis, as well as arrests the cell cycle. BAG-1 has been suggested to function as a molecular switch that encourages cells to proliferate in normal conditions but become quiescent under a stressful environment [19].

The regulation by BAG-1 of cell growth and survival is BAG-domain dependent. Human breast cancer cells ZR-75-1 that were stably transfected with BAG-1 or BAG-1L mutants that lack the BAG domain showed retarded growth rates and smaller tumor size [25]. The anti-apoptotic effects of the BAG-family proteins may be caused by the interaction of the BAG domain with Hsc70/Hsp70. BAG-family proteins act as co-chaperones and indirectly regulate the Hsc70/Hsp70-mediated protein refolding or protein degradation process, which may result in elevated levels of oncogenic proteins inside the cells.

BAG-1 and BAG-3/CAIR-1 have been shown to interact with the anti-apoptotic protein Bcl-2 by yeast two-hybrid, immunostaining and by co-immunoprecipitation [[2,10] Doong and Kohn unpublished observations]. In addition, green fluorescent protein (GFP)-BAG-1 fusion protein was used to demonstrate that BAG-1 and Bcl-2 are co-localized at organelles resembling mitochondria [10]. Although the precise binding site of the BAG protein to Bcl-2 has not yet been identified, it may be located at the carboxyl terminus of the BAG protein since Bcl-2 was pulled down by GST fusion protein containing the carboxyl terminus of BAG-3/CAIR-1 [2]. The C-terminal BAG domain is a possible site of interaction with Bcl-2 because ATP disrupts the binding of BAG-1 to Bcl-2. It is a provocative hypothesis that the binding of Bcl-2 to BAG proteins is through the BAG domain and that this interaction may be mediated by Hsc70/Hsp70. Supra-additive anti-apoptotic effects of BAG-1 or BAG-3/CAIR-1 were shown with Bcl-2. A strong anti-apoptotic effect was observed when the cells over-expressed both BAG-1 and Bcl-2 [1]. Over-expression of BAG-3/CAIR-1 also synergizes the anti-apoptotic effect of Bcl-2 [2]. A complete understanding of the binding of Bcl-2 to BAG-family proteins may further illustrate how these proteins collaborate with each other in the anti-apoptotic pathways.

BAG-4/SODD is associated with the cytoplasmic domain of TNF-R1 and death receptor-3 (DR-3). Recent data suggest that TNF-R1 contains an ATPase domain located in the N-terminus of the receptor's death domain and that ATP can regulate the interaction between BAG-4/SODD and TNF-R1 [14]. Increasing concentrations of Hsc70/Hsp70 have been shown to disrupt the binding of BAG-4/SODD to TNF-R1 ex vivo, indicating that Hsc70/Hsp70 may compete with TNF-R1 for BAG-4/SODD binding. BAG-4/SODD binds TNF-R1 under normal physiological conditions. When the cells are stimulated with TNF-α BAG-4/SODD dissociates from TNF-R1, allowing TRADD and TRAF2 to bind to the activated TNF-R1, which then leads to subsequent apoptosis signaling. BAG-4/SODD therefore, functions as an anti-apoptotic protein, as the binding of BAG-4/SODD to TNF-R1, prevents the ligand-independent oligomerization and activation of the receptor. The over-expression of BAG-4/SODD in various cancer cell lines has also been shown to suppress TNF-induced NF-*k*B activation and cell death [12,26]. The binding of BAG-4/SODD to TNF-R1 requires ATP. TNF-R1 is self-aggregated in ATP-depleted cells and BAG-4/SODD can disassemble TNF-R1 aggregates in vitro only when ATP is presented [14]. Hence, BAG-4/SODD may act as a nucleotide exchange factor, much like BAG-1 does for Hsc70/Hsp70.

4. Additional domains

BAG-family proteins also interact with other proteins through their N-terminal domains or motifs. All four BAG-1 isoforms and BAG-6/Scythe have an ubiquitin-like (UBL) domain at their amino-terminus (Fig. 1). BAG-1 and BAG-1M have been demonstrated to co-immunoprecipitate with the 20S core and the 19S subunit of the proteasome [27]. However,

no function for this interaction has been demonstrated. The BAG-1 proteins may serve as adapter molecules linking the proteasome to the Hsp70 chaperone complex. BAG-1 proteins also cooperate with C-terminus of Hsc70-interacting protein (CHIP), a newly identified ubiquitin E3 ligase, in the Hsc70/Hsp70-mediated protein degradation process [28–32]. The UBL domain of BAG-6/Scythe has not yet been linked to partner proteins or function.

A nuclear localization signal (NLS) normally indicates that the protein of interest can enter the nucleus. Generally, NLSs have been categorized as either monopartite, containing a cluster of basic amino acids, or bipartite, containing two basic regions separated by a variable spacer region [33,34]. Two of the BAG-family proteins, BAG-1 and BAG-6/Scythe, contain a NLS and have been shown to localize to the nucleus. BAG-1L has a monopartite NLS located at the N-terminus. Additionally, all of the BAG-1 isoforms contain a bipartite NLS at the C-terminus upstream of the BAG domain [35,36]. Although BAG-1M, -1, and -1S are considered cytoplasmic proteins, BAG-1 and -1M can be found in the nucleus depending on the cell type and whether or not the cells are exposed to stress conditions [10]. This phenomenon may be attributed to BAG-1 and -1M being transported into the nucleus via another protein or to the bipartite NLS.

BAG-6/Scythe also contains a C-terminal bipartite NLS upstream of the BAG domain. Site-directed mutagenesis of the BAG-6/Scythe NLS resulted in complete nuclear exclusion of the protein [17]. BAG-6/Scythe has been hypothesized to form a complex with Hsc70/Hsp70 and an unidentified apoptosis-inducing factor in the nucleus. When the cells are exposed to a stressful environment, another potent apoptosis-inducing protein, *reaper*, binds BAG-6/Scythe and causes the dissociation of the unidentified apoptosis-inducing factor from BAG-6/Scythe. The unidentified factor translocates from the nucleus to the cytoplasm and then induces the release of cytochrome *c* from the mitochondria and subsequent apoptotic events [16,23]. Thus, the nuclear localization of BAG-6/Scythe is necessary for its involvement in cell anti-apoptotic mechanisms.

The proline rich repeat, PXXP, is a canonical binding domain for partner proteins containing an Src homology 3 (SH3) motif [37–39]. BAG-3/CAIR-1 contains several tandems PXXP repeats with a total length of approximately 110 amino acids. Phospholipase C-γ (PLC-γ), an enzyme containing a SH3 domain, was found to co-immunoprecipitate with BAG-3/CAIR-1 and that this interaction was strictly through the PXXP-SH3 domain [11]. PLC-γ was pulled down from whole cell lysates using GST fusion proteins containing the PXXP repeats of BAG-3/CAIR-1. Similarly, GST–PLC-γ–SH3 constructs were able to pull down BAG-3/CAIR-1. Epidermal growth factor (EGF) was found to regulate the binding of PLC-γ to BAG-3/CAIR-1 by stimulating the release of PLC-γ from BAG-3/CAIR-1. The dissociation of PLC-γ occurs rapidly after EGF stimulation, and the released PLC-γ is then activated by the EGF receptor [11]. The subsequent activation of PLC-γay stimulate cell migration or promote cell survival by increasing cell proliferation [40]. Interestingly, the latest findings suggest that PLC-γ also participates in anti-apoptotic pathways and is necessary for the prevention of cell death induced by loss of extracellular matrix adhesion [41]. Other BAG-family proteins, such as BAG-6/Scythe, also contain a proline-rich region, however, no specific binding partners have been identified.

The WW domain is known to interact with PXXP regions. This domain consists of two tryptophan residues spaced 20–22 amino acids apart in a β-sheet structure [42]. BAG-3/CAIR-1 is the only BAG-family protein that contains a WW domain [4]. The function of the N-terminal WW domain remains unidentified. Other WW domain-containing proteins, such as dystrophin, utrophin and caveolin-3, have been found to play roles in connecting the cell membrane with the cytoskeletal structure [43]. This interaction for BAG-3/CAIR-1 is provocative given its binding to PLC-γ and anti-apoptotic activity.

BAG-1 proteins form complexes with protein tyrosine kinase receptors including the platelet-derived growth factor (PDGF) and hepatocyte growth factor (HGF) receptors, and several nuclear hormone receptors, such as androgen receptor (AR) and vitamin D receptor (VDR) [44–46]. BAG-1L binds AR and enhances the transactivation of androgen response element in PC3 prostate cancer cells. A carboxyl terminus deletion mutant of BAG-1L fails to co-immunoprecipitate with AR suggesting that the AR binding domain is located somewhere at the carboxyl

terminus of BAG-1L. On the contrary, BAG-1L binds VDR through its amino-terminus. BAG-1M and BAG-1 do not interact with VDR because they lack the first 71 (for BAG-1M) and first 145 (for BAG-1) N-terminal amino acids of BAG-1L. The binding of BAG-1L to VDR blocks vitamin D-induced transcriptional activation and apoptosis, resulting in increased cell growth rate in BAG-1L over-expressed cells [44]. These findings indicate that BAG-family proteins are capable of interacting with other proteins through their N-terminal domains and motifs, and may function as adapter molecules by recruiting molecular chaperones to target proteins and alter the functions of these target proteins. Hence, BAG-family proteins participate in a wide variety cellular processes including protein degradation, cell proliferation, migration and apoptosis.

5. BAG-family proteins and cancers

BAG-family proteins express differentially in a variety of human cancers and tumor cell lines, notably leukemias and breast, prostate, and colon cancers [10,28,47–50]. BAG-family proteins have been shown to regulate cell growth and block apoptosis [10,25]. Over-expression of either BAG-1 or BAG-1L can enhance survival of serum-deprived breast cancer cells. Serum-deprived BAG-1 or BAG-1L stably transfected ZR-75-1 cells remained viable (100%), while 80% of the neo control cells died after 9 days [25]. A marked increase in caspase activity was found in the neo control but not in BAG-1 or BAG-1L over-expressed cells, indicating that cell death was caused by apoptosis. In contrast, the C-terminal deletion mutants of either BAG-1 or BAG-1L failed to show protective effects. Tumor xenograft studies show similar results. When ZR-75-1 cells were injected into mammary fat pads of female nude mice, cells over-expressing BAG-1 or BAG-1L formed 1.4–1.6 fold larger tumors compared with neo control [25].

High level expression of BAG-1 isoforms has been found in human breast cancers [28,47,48,50]. BAG-1 was expressed in 147 of 160 cases (92%) of invasive breast cancers shown by immunostaining. Most of the tumors exhibited cytoplasmic BAG-1, while a small portion showed nuclear expression [50]. In a separate study, Tang et al. reported that BAG-1 was expressed in 77.1% of 140 breast carcinomas examined by immunostaining. Using multivariate analysis, BAG-1 expression was found to be significantly associated with shorter disease-free ($P = 0.0052$) and overall survival ($P = 0.0033$) [47]. This result, however, is contradictory to a recent study by Turner et al., who reported that cytoplasmic BAG-1 was up-regulated in 65% of 122 invasive breast cancers examined [48]. Elevated BAG-1 expression was significantly associated with longer disease-free ($P = 0.005$) and overall survival ($P = 0.001$) using multivariate analysis [48]. This surprising inconsistency may be caused by several factors. For instance, Turner et al. studied the early stages (I and II) of breast cancer patients rather than patients with metastatic disease or unknown stages. Additionally, Turner et al. correlated nuclear and cytoplasmic BAG-1 staining separately, instead of combining them, with patient outcome. Whether or not BAG-1 expression can be used as a biomarker to predict long-term survival requires for further investigation.

Over-expression of BAG-1 was also found to enhance cell migration and survival in two gastric cancer cell lines [51]. Over-expression of BAG-family proteins has been shown to increase resistance to chemotherapy. BAG-1 inhibits retinoic acid-induced apoptosis in human MCF-7 and ZR-75-1 breast cancer cells by direct binding to the retinoic acid receptor. This inhibition of cell death has been linked to retinoid resistance in cancers and other diseases [9]. Moderate to strong levels of the BAG-3/CAIR-1 and BAG-4/SODD mRNA transcript were detected in a variety of pancreatic cancer patient samples as well as in some human pancreatic cancer cell lines, whereas, weak levels were reported for normal pancreas samples [40]. Increased levels of BAG-4/SODD have been shown to be related to the resistance of pancreatic cancer cells to TNF-α. However, no cancer patient cohorts have been examined for BAG-6/Scythe.

6. Conclusions

In summary, BAG-family proteins may be related to tumorgenecity and can inhibit apoptosis through several mechanisms, most of which require a func-

tional BAG domain. This protein family presents a series of provocative biochemical and cellular events ripe for targeting for novel molecular therapeutics.

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