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p53-inducible human homologue of *Drosophila seven in absentia* (Siah) inhibits cell growth: suppression by BAG-1

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The *Drosophila seven in absentia* (*sina*) gene is required for R7 photoreceptor cell formation during *Drosophila* eye development, where it functions within the Ras/Raf pathway and targets other proteins for degradation via associations with a ubiquitin-conjugating enzyme. Recently, a mammalian *sina* homologue was reported to be a p53-inducible gene in a myeloid leukemia cell line. To explore the function of human SINA-homologous (Siah) proteins, expression plasmids encoding Siah-1A were transiently transfected into 293 epithelial cells and GM701 fibroblast cells, resulting in growth arrest without induction of apoptosis. We discovered that BAG-1, a ubiquitin-like Hsp70/Hsc70-regulating protein, is a negative regulator of Siah-1A. Siah-1A was identified as a BAG-1-binding protein via yeast two-hybrid methods. Specific interaction of BAG-1 with Siah-1A was also demonstrated by *in vitro* binding experiments using glutathione *S*-transferase fusion proteins and co-immunoprecipitation studies. Siah-1A-induced growth arrest in 293 and GM701 cells was abolished by co-transfection of wild-type BAG-1 with Siah-1A but not by a C-terminal deletion mutant of BAG-1 that fails to bind Siah-1A. Over-expression of BAG-1 significantly inhibited p53-induced growth arrest in 293 cells without preventing p53 transactivation of reporter gene plasmids. BAG-1 also prevented growth arrest following UV-irradiation-induced genotoxic injury without interfering with accumulation of p53 protein or *p21^{waf-1}* expression. BAG-1 functions downstream of p53-induced gene expression to inhibit p53-mediated suppression of cell growth, presumably by suppressing the actions of Siah-1A. We suggest that Siah-1A may be an important mediator of p53-dependent cell-cycle arrest and demonstrate that Siah-1A is directly inhibited by BAG-1.

Keywords: BAG-1/p21^{waf-1}/p53/Siah/*sina*

Introduction

Siah is the vertebrate homologue of the protein encoded by the *Drosophila seven in absentia* (*sina*) gene, which is required for formation of the R7 photoreceptor cells in the developing eye of the fly (Carthew and Rubin, 1990). The murine homologues of *sina* (*Siah-1A*, *Siah-1B* and *Siah-2*) are widely expressed in various tissues of the embryo and adult (Della *et al.*, 1993). The murine *Siah-1A* and *-1B* genes encode highly homologous proteins that share ⩾98% amino acid sequence homology with each other, and >95 and >68% homology with their human and *Drosophila* counterparts, respectively. In *Drosophila*, the SINA protein has been shown to interact with a ubiquitin-conjugating enzyme (UBCD1) and assists with the proteasome-dependent degradation of a transcription factor (Tramtrack) which suppresses R7 cell differentiation (Li *et al.*, 1997; Tang *et al.*, 1997). The human Siah-1 and Siah-2 proteins have also been shown to bind ubiquitin-conjugating enzymes (Hu *et al.*, 1997). Though the function of Siah-family proteins in vertebrates remains unknown, the murine *Siah-1B* was recently identified by differential cDNA display methods among a group of genes induced during p53-induced apoptosis and G_1-arrest in murine M1 myeloid leukemia cells (Amson *et al.*, 1996).

The tumor suppressor p53 has multiple functions in cells, including an ability to induce cell-cycle arrest in essentially all types of cells and to trigger apoptosis in some specific cell lineages and cellular contexts (Hartwell and Kastan, 1994; Reed *et al.*, 1996; Hall and Lane, 1997). The cell-cycle arrest induced by p53 can occur at either the G_1/S-phase or G_2/M-phase boundaries depending on cellular background and other circumstances (el-Deiry *et al.*, 1993; Harper *et al.*, 1993; Agarwal *et al.*, 1995; Guillouf *et al.*, 1995; Stewart *et al.*, 1995; Elledge, 1996; Levine, 1997). p53 has been shown to transactivate or transrepress a wide variety of target genes, including some associated with growth arrest and apoptosis, such as *p21^{waf-1}*, *Bax*, *Bcl-2*, *IGF-II*, *insulin receptor*, *IGF-I receptor* and *IGF-BP3* (el-Deiry *et al.*, 1993; Miyashita *et al.*, 1994; Buckbinder *et al.*, 1995; Miyashita and Reed, 1995; Webster *et al.*, 1996; Werner *et al.*, 1996; Zhang *et al.*, 1996; for reviews see Hartwell and Kastan, 1994; Elledge, 1996; Reed *et al.*, 1996; Hall and Lane, 1997; Levine, 1997). To date, investigations of the roles of these p53-regulated genes using targeted gene disruption (knock-out) approaches in mice and cell lines have all suggested that no single gene completely accounts for the ability of p53 to induce cell-cycle arrest and apoptosis (Deng *et al.*, 1995; Brown *et al.*, 1997; Yin *et al.*, 1997). These results have again demonstrated that cellular context plays an important role in determining which specific p53 target genes are relatively more or less required for mediating the actions of this tumor suppressor.

BAG-1 is a widely expressed protein that was first discovered by virtue of its ability to bind to and collaborate with Bcl-2 in suppressing cell death (Takayama *et al.*, 1995). The murine and human BAG-1 proteins (also known as RAP46) contain a domain with strong sequence homology to ubiquitin, but otherwise do not share

similarity with other known proteins (Takayama *et al.*, 1995, 1996). Since its initial discovery as a Bcl-2-binding protein, however, BAG-1 has been reported to interact with several steroid hormone receptors, the serine/threonine-specific protein kinase Raf-1 and some tyrosine kinase growth factor receptors [hepatocyte growth factor (HGF) receptor and platelet-derived growth factor (PDGF) receptor; Zeiner and Gehring, 1995; Bardelli *et al.*, 1996; Wang *et al.*, 1996]. Recently, we and others have determined that BAG-1 forms tight complexes with Hsp70/Hsc70-family proteins and modulates their chaperone activity (Höhfeld and Jentsch, 1997; Takayama *et al.*, 1997; Zeiner *et al.*, 1997). Thus, BAG-1 is probably a novel component of the chaperone system and presumably exerts its effects on various target proteins by recruiting Hsp70/Hsc70-family proteins to them.

We present evidence here that the p53-inducible Siah protein is a negative regulator of cell proliferation. The findings suggest that p53-mediated induction of Siah could represent an alternative to the $p21^{waf-1}$ pathway for cell-cycle arrest, and therefore suggest a redundant mechanism by which p53 interferes with cell proliferation. In addition, we show that the BAG-1 protein interacts with Siah-1A. BAG-1 abrogates Siah-1A-induced growth inhibition and can interfere with p53-mediated growth arrest. The discovery of a physical and functional connection between Siah-1A and BAG-1 therefore extends the range of potentially oncogenic activities of BAG-1 to suppression of p53-dependent pathways for cell growth inhibition.

Results

Identification of human Siah-1A as a BAG-1-binding protein

During attempts to identify proteins that associate with the BAG-1 by yeast two-hybrid cDNA library screening, we discovered a human SINA homologue. From a pool of 23 candidate clones, four corresponded to overlapping clones encoding polypeptides of 276 (clone 21) and 262 (clones 9, 17 and 26) amino acids in length which shared >95% homology at the nucleotide level with murine cDNA sequences encoding Siah-1A and Siah-1B (Della *et al.*, 1993). Subsequently, a human homologue of Siah-1 was reported which is essentially identical to the cDNAs identified by two-hybrid screening with BAG-1 as a bait (Nemani *et al.*, 1996). The longest cDNAs cloned during two-hybrid screening encompasses residues 22–298 of the predicted 298 amino acid human Siah-1 protein, whereas the shortest encoded residues 36–298 (Figure 1C). Like its murine and fly counterparts, the predicted human Siah protein contains a putative RING-finger domain, cysteine-rich region and nuclear localization signal sequence (Figure 1C). In the region corresponding to residues 17–298, the human Siah-1 protein differs from the murine Siah-1A homologue by only one amino acid, at position 132 where a glutamic acid to aspartic acid substitution occurs. In this same region, the human Siah-1 protein differs from murine Siah-1B at six residues, prompting us to designate the BAG-1-binding protein obtained from two-hybrid screening as Siah-1A as opposed to Siah-1B.

Specific interaction of Siah-1A with BAG-1

Siah-1A exhibited specific interactions with the BAG-1 protein in two-hybrid assays (Table I). Interestingly, Siah also appeared to interact with itself, suggesting that it can homodimerize or homo-oligomerize. To further confirm the interaction of Siah-1A with BAG-1, *in vitro* binding assays were performed using *in vitro* translated, ^{35}S-labeled BAG-1 and affinity-purified glutathione *S*-transferase (GST)–Siah-1A fusion protein. ^{35}S-BAG-1 bound to GST–Siah-1A but exhibited little or no tendency to bind to GST–CD40 (Figure 1A) and several other GST control proteins including GST–Bax, GST–TNF-RI and GST–XIAP (data not shown). Furthermore, a GST–Siah-1A fusion protein interacted with *in vitro* translated, ^{35}S-labeled Siah, implying that Siah can indeed form homodimers or homo-oligomers. In contrast, GST–Siah-1A did

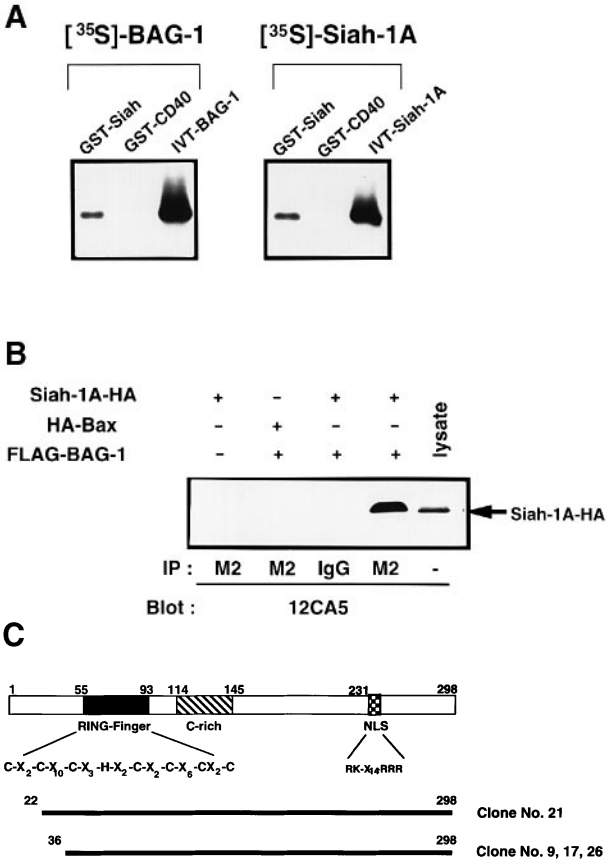

Fig. 1. *In vitro* and *in vivo* interaction between BAG-1 and Siah. (**A**) GST fusion proteins (10 μg) were immobilized on glutathione–Sepharose and incubated with 10 μl of ^{35}S-labeled, *in vitro* translated (IVT) BAG-1 (left) or Siah (right). After extensive washing, beads were boiled in Laemmli sample buffer and eluted proteins were analyzed by SDS–PAGE (12% gels) followed by detection by fluorography. In the last lanes, 1 μl of *in vitro* translated (IVT) proteins were run directly in the gel as a control. The control GST fusion protein represents the cytosolic domain of CD40 (Sato *et al.*, 1995). (**B**) 293 cells (5×10^6) were transiently transfected with the indicated combinations of expression vectors encoding FLAG-tagged BAG-1, HA-tagged Siah or HA-Bax (lane 1–4). After 48 h, lysates were prepared and immunoprecipitated with anti-FLAG monoclonal antibody M2-conjugated to agarose. Immune complexes were analyzed by SDS–PAGE/immunoblotting using anti-HA monoclonal antibody. In the last lanes, 10 μl of lysate was also loaded directly in the gel without immunoprecipitation as a control. (**C**) Diagram of the putative domain structure of human Siah-1A. Shown are the positions of the conserved RING-finger domain, cysteine-rich region and predicted nuclear localization signal sequence. The regions encoded by the partial cDNAs that were obtained by yeast two-hybrid screening are indicated below the diagram.

Table I. Specific interactions of Siah with BAG-1

LexA	B42	LEU	β-Gal
BAG-1	Siah	+	+
Bax	Siah	−	−
Fas	Siah	−	−
Ras	Siah	−	−
Lamin-C	Siah	−	−
empty	Siah	−	−
Siah	BAG-1	+	+
Siah	Bax	−	−
Siah	Ras	−	−
Siah	Siah	+	+

Three micrograms of plasmids producing LexA DNA-binding domain fusion proteins (listed at left) were co-transformed with 3 μg of pJG4-5 plasmid producing B42 transactivation domain fusion proteins (listed at right) into EGY48 strain yeast. Transformed cells were grown on semi-solid media lacking leucine, or containing leucine as a control which resulted in equivalent amounts of growth for all transformants (data not shown). Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+). β-galactosidase activity of each colony was tested by filter assay and scored as blue (+) or white (−) after 60 min.

not bind *in vitro* translated ^{35}S-Bax, indicating that the homodimerization detected for Siah-1A is specific (data not shown). Recently, the *Drosophila* SINA protein was also reported to homodimerize (Li *et al.*, 1997; Tang *et al.*, 1997).

To examine whether the interaction of Siah-1A with BAG-1 occurs in cells, we performed co-immunoprecipitation experiments. An expression plasmid encoding FLAG epitope-tagged BAG-1 was transfected into 293 cells alone or in combination with plasmids encoding either hemagglutinin (HA) epitope-tagged Siah-1A, or HA-Bax as a negative control. The resulting cell lysates were immunoprecipitated using a monoclonal antibody specific for the FLAG epitope (M2) and associated Siah-1A-HA protein was detected by immunoblotting using an anti-HA monoclonal antibody (12CA5). Siah-1A-HA co-immunoprecipitated with FLAG-BAG-1 whereas HA-Bax did not (Figure 1B).

Analysis of BAG-1/Siah-1A interaction

To explore the regions of BAG-1 responsible for interaction with Siah-1A, we prepared a series of BAG-1 deletion mutants that were expressed in yeast as fusion proteins with a LexA DNA-binding domain (Figure 2A). Western blotting using an anti-LexA antiserum confirm production of all BAG-1 proteins at comparable levels (data not shown). An N-terminal deletion mutant of BAG-1 (amino acids 90–219) which removed the ubiquitin-like domain retained Siah-binding activity. In contrast, a C-terminal deletion mutant of BAG-1 (amino acids 1–172) failed to interact with Siah-1A, as did a double-truncation mutant of BAG-1 (90–172) which lacked both the N- and C-terminal regions. Similar results were obtained by *in vitro* binding assay, using GST–BAG-1, GST–BAG-1 (ΔN) and GST–BAG-1 (ΔC) fusion proteins (Figure 2B). These observations demonstrate that the C-terminal portion of BAG-1 (residues 173–219) is required for binding to Siah-1A. It is known that this same C-terminal region of BAG-1 is also essential for association with Hsp70/Hsc70 (Takayama *et al.*, 1997),

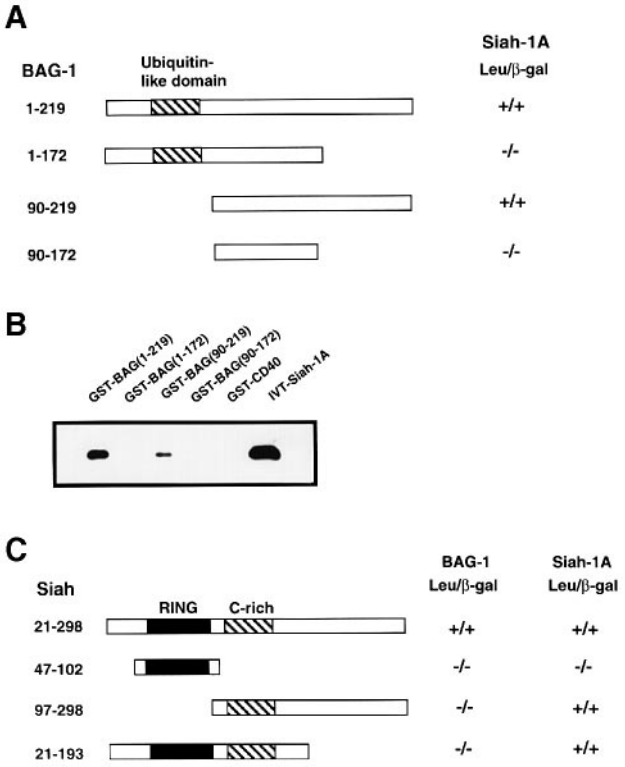

Fig. 2. Mapping of BAG-1–Siah and Siah–Siah interaction domains. (**A**) Expression plasmids encoding wild-type BAG-1 and the indicated BAG-1 deletion mutants fused to the LexA DNA-binding domain were co-transformed into yeast EGY48 cells with a plasmid encoding B42 transactivation domain–Siah (22–298) fusion protein. Transformed cells were grown on semi-solid media lacking leucine or containing leucine as a control. Plasmid combinations that resulted in growth on leucine-deficient media within 4 days were scored as positive (+). β-galactosidase activity for each colony was tested by filter assay and scored as blue (+) versus white (–) (β-gal) based on 1 h of color development. (**B**) GST fusion proteins (10 μg) containing wild-type BAG-1 and the indicated BAG-1 deletion mutants were immobilized on glutathione–Sepharose and incubated with ^{35}S-labeled Siah. After extensive washing, beads were boiled in Laemmli buffer and eluted proteins were analyzed by SDS–PAGE (12% gels) and detected by fluorography. *In vitro* translated (IVT) Siah protein (1 μl) was run directory in the gel as a control. A GST fusion protein representing the cytosolic domain of CD40 protein was included as a control. Production of mostly intact GST–BAG-1 wild type and mutant fusion proteins was verified by Coomassie staining (data not shown). (**C**) Expression plasmids encoding the indicated Siah-1A deletion mutants fused to the B42 transactivation domain were co-transformed into yeast EGY48 cells with LexA DNA-binding domain–BAG-1 or LexA–Siah (22–298) expression plasmids. Yeast two-hybrid assays were performed as in (A).

steroid hormone receptors (Zeiner and Gehring, 1995), Raf-1 (Wang *et al.*, 1996) and the HGF receptor (Bardelli *et al.*, 1996). The observation that a C-terminal truncation mutant of BAG-1 failed to bind Siah provides further evidence of the specificity of these results.

We examined the regions in Siah-1A which are required for binding to BAG-1 and for homodimerization using yeast two-hybrid assays. All Siah-1A deletion mutants tested failed to interact with BAG-1 (Figure 2C), suggesting that two or more separate sites on Siah-1C may be required for BAG-1-binding or that these mutants fail to fold properly for presentation of BAG-1 binding structures. In contrast, Siah-1A mutants lacking the RING domain or most of the C-terminal unique domain retained

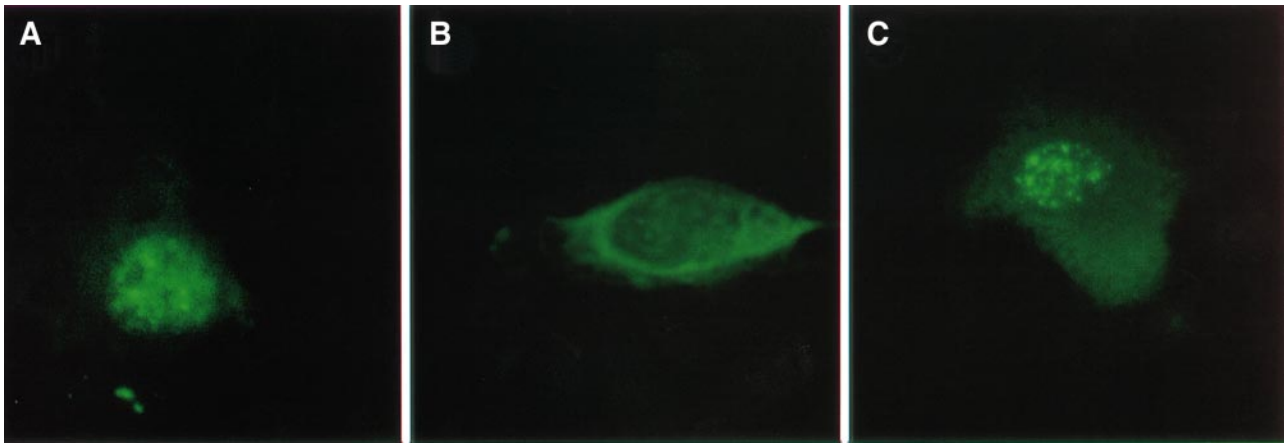

Fig. 3. Immunofluorescence microscopic evaluation of locations of BAG-1 and Siah-1A proteins. 293 cells were transiently transfected with pCI–FLAG-Siah (**A**), pCI–FLAG-BAG-1 (**B**) and pCI–FLAG-BAG-1 in combination with pCI–Siah (**C**). After 48 h, cells were immunostained with anti-FLAG monoclonal antibody M2 and FITC-conjugated goat anti-mouse immunoglobulin and analyzed by fluorescence microscopy (~800× original magnification).

the ability to bind to Siah-1A in yeast two-hybrid assays (Figure 2C). These results imply that the region of Siah-1A corresponding to amino acids 102–193 where the cysteine-rich domain resides is involved in Siah-1A homodimerization.

Siah-1A alters the subcellular location of BAG-1 in cells

If BAG-1 and Siah-1A associate in intact cells, we reasoned that their co-expression might affect the intracellular locations of these proteins in cells. To test this hypothesis, FLAG-tagged BAG-1 and Siah-1A proteins was co-expressed in GM701 cells and their distribution patterns were determined by indirect immunofluorescence. When a cDNA encoding nearly full-length Siah-1A (22–298) with a FLAG epitope-tag was transfected into GM701 cells by itself and then detected with an antibody directed against the FLAG epitope by immunofluorescence microscopy, the Siah-1A protein was predominantly present in the nucleus, though some weak cytosolic immunofluorescence was also seen (Figure 3). Consistent with these results, the *Drosophila* SINA protein was reported to display a very similar subcellular distribution (Li *et al.*, 1997; Tang *et al.*, 1997). Moreover, the immunolocalization of Siah-1A in the nucleus suggested a punctate pattern, implying association with unidentified substructures in the nucleus. When a FLAG-BAG-1 protein was expressed in these cells by itself, immunofluorescence was found in both the cytosol and nucleus, but was greater in the cytosol (Figure 3B). In contrast, cells co-transfected with FLAG-BAG-1 and untagged Siah-1A expression plasmids displayed a predominantly nuclear localization of the FLAG-BAG-1 protein (Figure 3C). In reciprocal experiments where FLAG-tagged Siah-1A was expressed with untagged BAG-1, the intracellular pattern of Siah-1A distribution was not substantially different from that seen in cells expressing FLAG-Siah-1A alone (data not shown). The specificity of these immunofluorescence data was verified by use of irrelevant control primary antibodies as well as by immunostaining with secondary antibody alone (data not shown). Taken together, these data suggest that Siah-1A can alter the intracellular location of BAG-1, targeting a portion of the BAG-1 molecules to the nucleus. As with Siah-1A, the BAG-1 immunofluorescence within the nuclei of cells that had been co-transfected with BAG-1 and Siah-1A was distributed in a punctate pattern (Figure 3C). The significance of this speckled nuclear immunolocalization pattern is unclear.

Siah-1A suppresses cell growth

Because Siah expression has been reported to be induced by p53, we investigated whether Siah-1A could induce either growth arrest or apoptosis. To explore this possibility, an expression plasmid encoding FLAG-Siah-1A was transiently transfected into 293 cells. These cells have been used extensively for studying apoptosis-inducing proteins because of their high efficiency of transient transfection (>90%). Two days after transfection, the numbers of viable and dead cells were determined by trypan blue dye exclusion assay. Siah-1A suppressed growth of 293 cells, without stimulating an increase in cell death (Figure 4A). Attempts to observe effects of Siah over-expression on cell death using other methods for detecting apoptotic cells such as DAPI fluorochrome staining and DNA-content analysis by flow cytometry also failed to demonstrate increased amounts of apoptosis in cultures of Siah-transfected cells. In contrast, over-expression of Bax, which is a p53-inducible pro-apoptotic gene (Miyashita and Reed, 1995), induced an ~5-fold increase in the number of dead cells as determined by trypan blue dye exclusion. The Siah-1A-mediated growth suppression was reversed by co-expression with BAG-1, but not by the C-terminal deletion mutant of BAG-1 which fails to bind Siah-1A. Immunoblot analysis confirmed that the expected ~34 kDa FLAG-Siah-1A (22–298), ~32 kDa FLAG-BAG-1 and ~27 kDa FLAG-BAG-1 (ΔC) (retains only amino acids 1–172) proteins were all produced at readily detectable levels in transiently transfected 293 cells (Figure 4B).

Siah-1A inhibits DNA synthesis in replicating cells

To further examine the ability of Siah-1A to inhibit cell growth, GM701 cells were transfected with plasmids encoding FLAG-tagged Siah and the relative amounts of

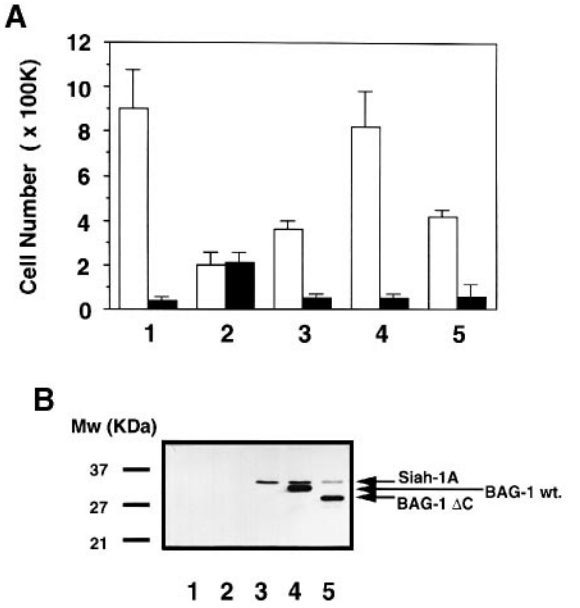

Fig. 4. Cell growth inhibition by Siah-1A in 293 cells. (**A**) 293 cells were transiently transfected with 10 μg of pCI–Neo (1), 5 μg of pCI–neo + 5 μg of pCI–Bax (2), 5 μg of pCI–Neo + 5 μg of pCI–FLAG-Siah (22–298) (3), 5 μg of pCI–FLAG-Siah (22–298) + 5 μg of pCI-FLAG–BAG (1–219) (4), or 5 μg of pCI–FLAG-Siah (22–298) + 5 μg of pCI–FLAG-BAG (1–172) (5). After 48 h, both floating and attached cells were collected and the numbers of viable and dead cells were estimated by trypan blue dye exclusion. Data (mean ± SE) are shown as the absolute number of living cells (open bar) and dead cells (closed bar) per 60 mm dish for three independent transfection experiments. (**B**) Immunoblot analysis confirmed production of expected FLAG-Siah (22–298) protein of ~34 kDa, FLAG-BAG-1 (1–219) protein of ~32 kDa and FLAG-BAG-1 (1–172) protein of ~27 kDa. Cell lysates were prepared from duplicated dishes of each transfection, normalized for total protein content (20 μg per lane), and analyzed by SDS–PAGE or immunoblotting using anti-FLAG monoclonal antibody with ECL-based detection.

DNA synthesis (^{3}H-thymidine incorporation into DNA) and the approximate percentage of S-phase cells [bromodeoxyuridine (BrdU)-labeling] were determined. As shown in Figure 5, Siah-1A expression reduced by over half on average: (i) the number of cells present in cultures 2 days after transfection; (ii) the relative amount of DNA synthesis (^{3}H-TdR incorporation); and (iii) the percentage of cells that were either in S-phase or had passed through S-phase (BrdU-positive). Propidium iodide-staining followed by flow-cytometric DNA content analysis also confirmed marked reductions in S-phase cells (58 ± 3% versus 39 ± 4%), with accumulations of the Siah-1A-expressing cells in both G_0/G_1 and G_2/M (data not shown). In contrast, these inhibitory effects of Siah-1A were largely reversed by co-expression of BAG-1 (Figure 5). Immunoblotting confirmed production of the Siah-1A and BAG-1 proteins and verified that BAG-1 did not inhibit production of the Siah-1A protein (data not shown).

BAG-1 interferes with p53-mediated cell growth suppression

Since Siah is a potential downstream mediator of the effects of p53 in cells, we examined the possibility that BAG-1 might prevent p53-induced cell-cycle arrest. For these experiments, an expression plasmid encoding wild-type p53 protein was transiently transfected into 293 cells, with or without a BAG-1-encoding plasmid. Over-

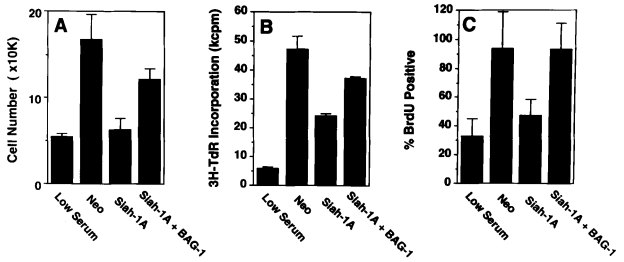

Fig. 5. Siah inhibits DNA synthesis in replicating GM701 cells. GM701 cells in DMEM containing 10% FCS were transiently transfected with pCI–neo (neo), pCI–FLAG-Siah or pCI-FLAG-Siah together with pRc/CMV–mBAG-1. (**A**) After 48 h, both floating and attached cells were recovered and viable cell numbers were estimated by trypan blue dye exclusion. As a control for growth inhibition, a duplicate culture of pCI–neo-transfected cells was switched to medium containing 0.1% FCS after 24 h. Alternatively, 24 h after transfection, ^{3}H-thymidine (**B**) or BrdU (**C**) was added and cultures were continued for 24 h. Data (mean ± SE) represent results from three independent transfection experiments. Transfecting BAG-1 expression plasmids by itself had no significant effects on cell growth or proliferation (data not shown).

expression of p53 in 293 cells (i) induced increases in Siah-1A mRNA levels, as determined by RNase protection assay (Figure 6A), and (ii) produced a significant reduction in cell growth (Figure 6B), but did not induce apoptosis, as determined by several criteria including DAPI-staining and trypan blue dye exclusion assays (data not shown). Co-transfection of BAG-1 and p53 expression plasmids revealed that BAG-1 can partially reverse the inhibitory effects of p53 on 293 cell growth in a dose-dependent fashion. The C-terminal deletion mutant of BAG-1, however, did not abrogate p53-mediated growth arrest. Immunoblot analysis verified production of the p53, BAG-1 and BAG-1 (ΔC) proteins (data not shown). Thus, BAG-1 was capable of interfering with the p53-induced inhibition of cell growth in 293 cells.

BAG-1 does not interfere with p53 protein induction or transactivation of target genes

Though we suspected that BAG-1 blocked p53-induced growth arrest indirectly by nullifying the actions of Siah-1A, it was possible that BAG-1 interfered directly with some aspect of p53 function. No interaction was detected between BAG-1 and p53 by co-immunoprecipitation or *in vitro* binding studies, indicating that BAG-1 does not bind directly to p53 (data not shown). Moreover, immunofluorescence microscopy analysis of p53 protein demonstrated that BAG-1 over-expression does not preclude entry of p53 into the nucleus of 293 cells (unpublished data). The effects of BAG-1 on p53 protein levels were explored using several stable transfectants, representing human tumor cell lines that contain wild-type (MCF7, ZR75-1) or mutant (293, Jurkat) p53 (Figure 7A). Over-expression of BAG-1 did not alter the basal levels of p53 protein in these cell lines, as demonstrated by immunoblotting with anti-p53 antibodies. Furthermore, BAG-1 over-expression did not interfere with the upregulation of p53 protein levels induced by γ-radiation and UV-radiation in MCF7 cells which express wild-type p53 (Figure 7B). In addition, BAG-1 did not prevent p53 from transactivating a reporter gene plasmid that contains a p53-response element (p53-RE) derived from the *BAX*

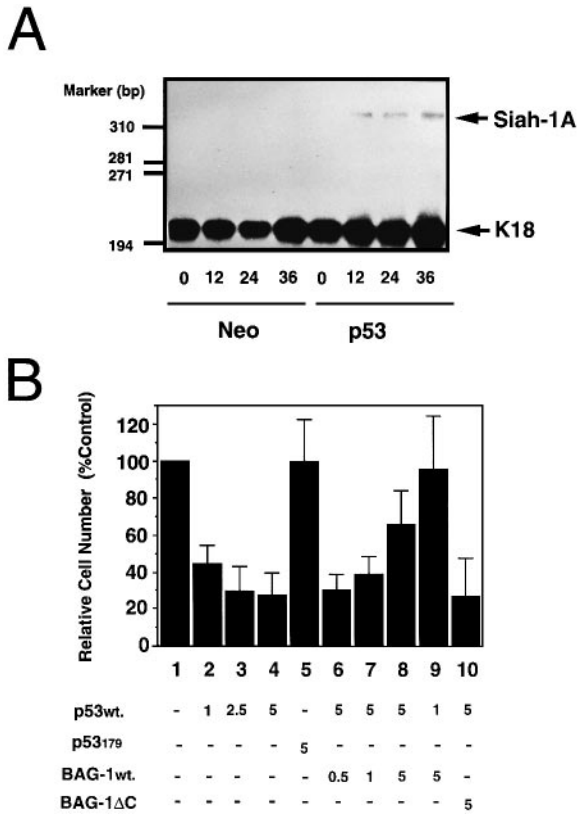

Fig. 6. (**A**) RNase protection analysis of Siah-1A RNA after p53 over-expression. 293 cells were transiently transfected with 10 μg of pCMV–Neo (Neo) of 10 μg of pCMVp53wt (p53). Total RNA was extracted from cells at 0, 12, 24 and 36 h after transfection and Siah-1A RNA expression was measured with the use of an RNase protection assay utilizing a probe containing 324 bp of Siah-1A cDNA. A probe containing 218 bp fragment of the human keratin 18 cDNA was used as internal control. (**B**) Effect of BAG-1 expression on p53-induced growth arrest. 293 cells were transiently transfected with expression vectors [1, 10 μg pCI–Neo; 2, 1 μg pCMVp53wt; 3, 2.5 μg pCMVp53wt; 4, 5 μg pCMVp53wt; 5, 5 μg pCMVp53$_{179}$; 6, 5 μg pCMVp53wt + 0.5 μg pCI–FLAG-BAG-1; 7, 5 μg pCMVp53wt + 1 μg pCI–FLAG-BAG-1; 8, 5 μg pCMVp53wt + 5 μg pCI–FLAG-BAG-1; 9, 1 μg pCMVp53wt + 5 μg pCI–FLAG-BAG-1; or 10, 5 μg pCMVp53wt + 5 μg pCI–FLAG-BAG-1 (1–172)]. After 48 h, viable cell numbers were estimated by trypan blue dye exclusion. Data (mean ± SE) are expressed as a percentage relative to pcI–Neo transfected cells.

gene promoter (Figure 7C). Taken together, these data argue that BAG-1 interferes indirectly with p53-mediated growth suppression by acting at a step distal to p53-inducible gene expression, as expected if BAG-1 operates as an inhibitor of Siah-1A.

BAG-1 prevents irradiation-induced cell-cycle arrest

To further explore the effects of BAG-1 on inhibition of p53-induced cell-cycle arrest, experiments were performed where UV-irradiation was used to induce accumulation of endogenous p53 protein in MCF7 breast cancer cells which had been stably transfected with either pcDNA3 control or pcDNA3–BAG-1 plasmids. Relatively low doses of UV-irradiation (10 J/m^2) were employed in these experiments, resulting in cell-cycle arrest without apoptosis. Increases in p53 and p21^{waf-1} protein levels were induced following UV-irradiation (Figure 8A). BAG-1 over-expression had no effect on these UV-induced

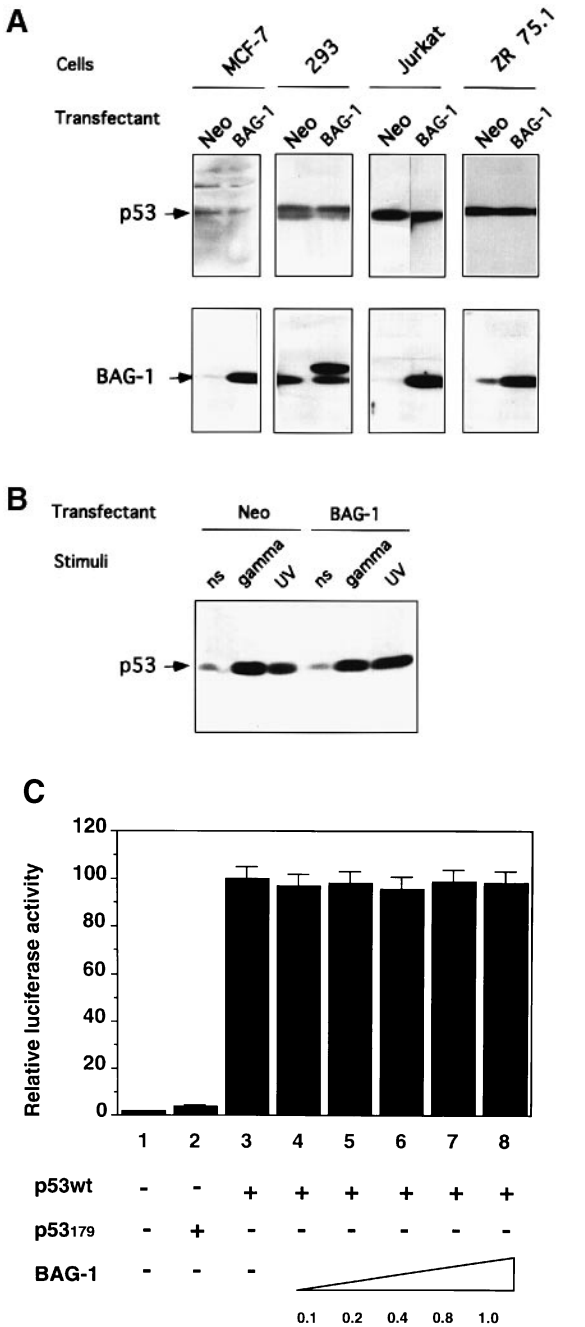

Fig. 7. BAG-1 does not interfere with p53 induction or p53-mediated gene transactivation. (**A**) Tumor cell lines [MCF-7, ZR 75.1, flag-tagged human BAG-1 (293) or murine BAG-1 (Jurkat)] were stably transfected with either control (Neo) or human BAG-1 expression plasmids. Total cell lysates were prepared, normalized for protein content (50 μg) and subjected to SDS–PAGE or immunoblot assay using either anti-p53 monoclonal antibodies (top) or a mAb specific for BAG-1. (**B**) MCF7–Neo and MCF7–BAG-1 transfectants were treated with 10 Gy γ-radiation or 10 J/m^2 UV-irradiation. After 4 h, total cell lysate was prepared (50 μg) and analyzed for p53 protein levels by immunoblotting. (**C**) 293 cells were transiently transfected by a standard calcium phosphate precipitate method with a p53-responsive reporter gene plasmid that contains a –645/–317 bp fragment from the *BAX* gene promoter cloned upstream of a luciferase reporter gene in pGL3. This reporter plasmid (0.4 μg) was co-transfected with 0.4 μg of pCMVp53wt and 0.2 μg of pCMV–β-galactosidase and various amounts of pRc/CMV–BAG-1 or pRc/CMV empty control plasmid (0–1 μg). Luciferase activity was measured in cell lysates 2 days later. Data shown are normalized relative to β-galactosidase, thus correcting for any differences in transfection efficiencies.

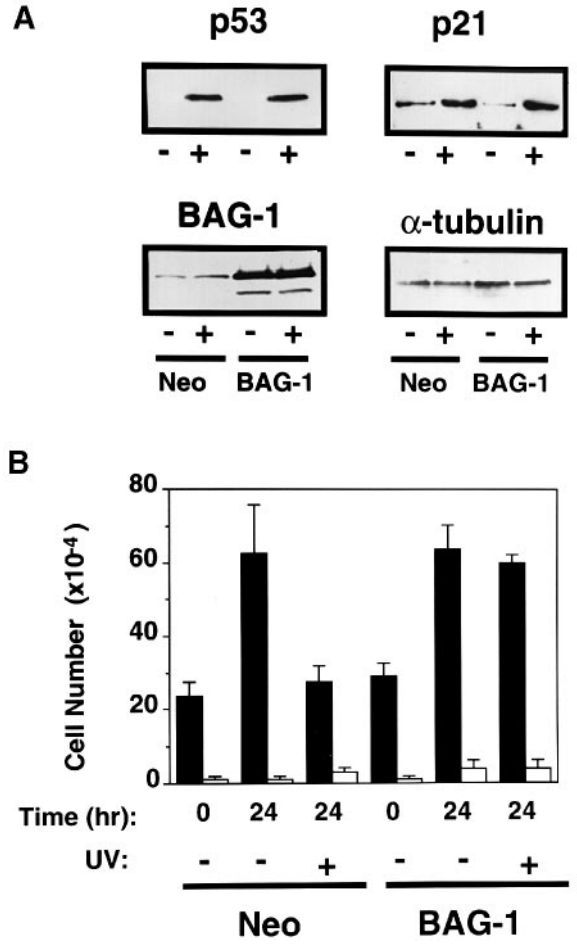

Fig. 8. BAG-1 prevents UV-induced arrest of cell growth without interferring with p53 or $p21^{waf-1}$ expression. MCF7 cells which had been stably transfected with either pcDNA3 ('NEO') or pcDNA3-BAG-1 plasmids ('BAG-1') were treated with 10 J/m^2 UV-irradiation. (**A**) Protein lysates were prepared from control or UV-irradiated cells after 24 h and 50 μg aliquots were analyzed by immunoblotting using antibodies specific for p53, $p21^{waf-1}$, BAG-1 or tubulin as a loading control. Detection was by an ECL-based method. (**B**) MCF7–NEO or MCF7–BAG-1 cells were seeded into 60 mm dishes at ~0.25×10^6 cells and then either recovered 1 h later and counted (0 h) or cultured for 24 h with or without prior exposure to 10 J/m^2 UV-irradiation. The numbers of viable (dark bars) and dead (open bars) cells were determined by trypan blue dye exclusion assay of the trypsin-recovered cells (mean ± SE; N = 3).

increases in p53 and $p21^{waf-1}$ (Figure 8A). Despite having no effects on irradiation-induced increases in p53, $p21^{waf-1}$ and Siah-1A expression, BAG-1 over-expression substantially interfered with UV-induced arrest of cell proliferation, restoring cell growth to nearly the levels of control non-irradiated MCF7 cells (Figure 8B). UV-irradiation induced comparable increases in Siah mRNA in MCF7 cells, regardless of BAG-1 protein levels (data not shown). We conclude therefore that BAG-1 blocks genotoxic-stress-induced cell-cycle arrest at a point downstream of p53 and without interfering with $p21^{waf-1}$ protein production.

Discussion

Recently, murine Siah-1B was identified by differential cDNA display among a group of genes induced early after activation of p53 in a leukemia cell line which undergoes G_1 arrest and apoptosis in response to p53 (Amson *et al.*, 1996). Based on this and other circumstantial evidence, it was suggested that the murine Siah-1B protein could promote apoptosis (Amson *et al.*, 1996; Nemani *et al.*, 1996). Similarly, expression of the human Siah-1A gene has been reported to be induced along the intestinal villi in association with growth arrest and apoptosis in this epithelium, implying a role for this member of the Siah-family in either cell-cycle inhibition or cell death (Nemani *et al.*, 1996). Until now, however, no direct evidence had been obtained to demonstrate an effect of Siah on either cell proliferation or survival. Here we confirm that p53 and genotoxic-stress injury induce expression of Siah-1A. Moreover, the results described here indicate that over-expression of the Siah-1A protein induced growth arrest of the human embryonic kidney line 293 and of human immortalized GM701 fibroblasts, but did not trigger apoptosis of these particular cells. It should be noted that because the ability of p53 to induce cell-cycle arrest versus apoptosis is highly context-dependent (Lowe *et al.*, 1993; Zhan *et al.*, 1994), we cannot exclude the possibility that Siah-1 might promote apoptosis in some other types of cells.

The $p21^{waf-1}$ protein is the best known inhibitor of the cell cycle whose expression is induced directly by p53 (el-Deiry *et al.*, 1993; Harper *et al.*, 1993). Upregulation of $p21^{waf-1}$ inhibits the function of cyclin-dependent kinases, particularly those that function during the G_1-phase of the cell cycle. However, mice deficient in the *WAF-1* gene (knock-outs) develop normally, and fibroblasts derived from p21-deficient mouse embryos are only partially defective in their ability to arrest cells in response to DNA damage (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). These observations suggest the existence of an alternative $p21^{waf-1}$-independent pathway through which p53 can suppress cell proliferation. Based on (i) the p53-inducibility of Siah-1A (Amson *et al.*, 1996); (ii) the ability of Siah-1A over-expression to inhibit cell growth; and (iii) the finding that BAG-1 can suppress both Siah-1A- and p53-induced growth arrest in transfection assays, Siah-family proteins should be considered candidates for mediators of this $p21^{waf-1}$-independent pathway.

Della *et al.* (1993) showed that the mouse genome contains a family of three Siah genes, called *Siah-1A*, *Siah-1B* and *Siah-2*. We also found several human cDNA fragments which have homologies to each of these members of mouse Siah family in the DDBJ/EMBL/GenBank database (Siah-1B: accession numbers T12004 and T12005; Siah-2: accession numbers R25253 and R61648), suggesting that the human genome also contains at least three Siah-family genes. It remains to be determined whether all of these Siah-family proteins can bind to BAG-1. The known members of the Siah family in humans, mice and *Drosophila* exhibit striking conservation of amino acid sequence homology over most of their length, only diverging significantly at their N-termini. Moreover, we have found evidence of potential plant homologues of the Siah protein in EST databases. These cDNA fragments include dbEST ID:725825 in rice, *Callus organium* and dbEST ID:439828 in *Arabidopsis thaliana*, which share significant homology with the RING-finger and cysteine-rich regions of Siah-1A, respectively (data

not shown). The high degree of amino acid sequence identity between *Drosophila* SINA and its vertebrate or plant counterparts suggests that Siah-family proteins may play a crucial role in cellular processes such as proliferation, survival or differentiation.

Recent genetic data indicate that *Drosophila* SINA functions downstream of the receptor tyrosine kinase *Sevenless* in the Ras-1/Raf-1 pathway (Carthew *et al.*, 1994). Moreover, it has been shown that Phyllopod, a nuclear factor whose expression is induced by *Sevenless* and the Ras-1/Raf-1/MAP kinase pathway (Chang *et al.*, 1995; Dickson *et al.*, 1995), interacts with SINA, forming a complex which is involved in upregulating transcription of the *prospero* gene in the eye. Recently, we reported that BAG-1 binds Raf-1 kinase and regulates its kinase activity (Wang *et al.*, 1996). This observation is consistent with the idea that some members of vertebrate Siah family may be involved in the Ras-1/Raf-1/MAP kinase pathway. However, the finding that BAG-1 enhances the activity of Raf-1, while inhibiting Siah-1A, makes it difficult to consolidate these results into a single model of Ras/Raf/MEK signaling.

Interestingly, SINA has recently been reported to bind to a ubiquitin-conjugating enzyme, UBCD1, and induce the proteasome-dependent degradation of a transcriptional repressor encoded by the *Tramtrack* gene (Li *et al.*, 1997; Tang *et al.*, 1997). The human Siah-1 and Siah-2 proteins have also been shown to bind a ubiquitin-conjugating enzyme, thus providing a mechanism for targeting interacting proteins for ubiquitination and proteolytic degradation via the proteosome (Hu *et al.*, 1997). BAG-1 contains a ubiquitin-like domain; this domain is not required for binding to Siah-1A but could conceivably be required for BAG-1-mediated inhibition of growth arrest by Siah-1A. Attempts to produce BAG-1 mutants lacking this domain, however, have led to unstable proteins, precluding exploration of the hypothesis (unpublished observations). Alternatively, BAG-1 may be a target for Siah-1A-mediated ubiquitination and degradation. However, we have not observed a reduction in BAG-1 protein levels in Siah-1A transfected cells, suggesting that this is not the case.

Based on the recently discovery that BAG-1 functions as a modulator of Hsp70/Hsc70-family proteins (Takayama *et al.*, 1997), we assume that BAG-1 targets these chaperones to Siah-1A, inducing conformational changes that directly or indirectly abrogate its growth arrest function. Interestingly, the C-terminal portion of BAG-1 that is required for Siah-1A-binding has also been shown to be necessary for the interaction of BAG-1 with Raf-1, HGF-R and PDGF-R (Bardelli *et al.*, 1996; Wang *et al.*, 1996). Conversely, the first ~90 amino acids of murine BAG-1, which contain a ubiquitin-like domain, are expendable for these same interactions. Thus, the domains within BAG-1 which are involved in Siah-1A-binding correlate with Hsp70/Hsc70-binding. We cannot, however, exclude the possibility that the C-terminal 47 amino acids of BAG-1 are merely required for its proper folding. Further studies are required to determine the role, if any, of Hsp70/Hsc70-family proteins in the functional and physical interactions of BAG-1 with Siah-1A.

The mechanism by which Siah-1A inhibits cell growth in mammalian cells is elusive. In the developing fly eye, SINA plays the role of a differentiation-inducing protein.

A

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Siah        57 CPVCFDYVLPPI-LQCQSGHLV-CSNCRPKLTCCPTCRG  93
C-IAP-1    571 CKVCMDKEVSVVFIPC--GHLVVCQECAPSLRKCPICRG 607
C-IAP-2    557 CKVCMDKEVSIVFIPC--GHLVVCKDCAPSLRKCPICRS 595
Consensus      C-VC-D---------C--GHLV-C--C-P-L--CP-CR-
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B

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Siah      114 CKYASSGCEITLPHTEKADHEELCEFRPYSCP 145
TRAF-4     83 CIHSEEGCRWSGPLRHLQGHLNTCSFNVIPCP 114
Consensus     C-----GC----P------H---C-F----CP
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Fig. 9. Sequence comparisons of Siah-family proteins. (**A**) The RING domains of human Siah, human c-IAP-1 and c-IAP-2 are aligned. (**B**) The cystine-rich region of human Siah is aligned with the CART domain of TRAF-4 (Régnier *et al.*, 1995).

However, since cell differentiation and growth arrest often are coordinately regulated, it is possible that SINA also contributes to R7 cell-growth inhibition in *Drosophila*. Conversely, it is conceivable that Siah-family proteins may participate not only in growth inhibition but also cell differentiation in some types of mammalian cells. The RING-finger of Siah-1A is most similar to those of the IAP-family proteins, c-IAP-1 and c-IAP-2 (Rothe *et al.*, 1995; Saurin *et al.*, 1996; Figure 9A), a group of anti-apoptotic proteins which bind to cell death proteases (Deveraux *et al.*, 1997). A DDBJ/EMBL/GenBank database search using the cysteine-rich region of Siah-1A revealed significant homology only to the cysteine-rich region of TRAF-4 (also known as CART; Régnier *et al.*, 1995), a member of a family of putative signal transducing proteins that bind to TNF family cytokine receptors. The relative positioning of the cysteine and histidine residues is identical in both proteins (Figure 9B). Interestingly, some IAP-family proteins have been reported to interact with same TRAF-family proteins, though not through the RING and cysteine-rich domains (Rothe *et al.*, 1995). The significance of these similarities of Siah to proteins implicated in TNF-family cytokine receptor signal transduction is unclear, but reports of involvement of ubiquitin-conjugating enzymes and the proteasome in both systems may provide some clues (Boldin *et al.*, 1995; Wright *et al.*, 1996). In contrast to the RING and cysteine-rich domains, database searches did not reveal any proteins with significant amino acid sequence homology to the C-terminal unique region (150–298) of human Siah-1A and its homologues. Identification of Siah-binding proteins analogous to *Phyllopod* and *Tramtrack* in *Drosophila* should provide additional insights into the mechanisms by which Siah-family proteins control cell growth in mammalian cells.

The relative importance of Siah in the overall tumor-suppressing function of p53 remains to be determined. Inasmuch as BAG-1 can abrogate the effects of Siah-1A on cell proliferation, the observation that BAG-1 inhibits p53-induced growth arrest suggests that Siah-1A or other Siah-family proteins are participants in p53-mediated inhibition of cell proliferation, at least in some circum-

stances. Moreover, because BAG-1 prevented p53-mediated growth arrest without interfering with $p21^{waf-1}$ expression, it is conceivable that Siah-family proteins provide an alternative p21-independent mechanism for p53-mediated suppression of cell growth. We cannot, however, exclude the possibility that BAG-1 interferes with p53-induced growth arrest by a combination of mechanisms, with abrogation of Siah-1A function representing only one of them. It is unlikely that the ability of BAG-1 to enhance the anti-apoptotic activity of Bcl-2 plays any role in the results reported here, since Bcl-2 has been shown to have no effect on p53-induced arrest of cell proliferation (Wang *et al.*, 1993; Miyashita *et al.*, 1994; Selvakumaran *et al.*, 1994). On the other hand, since Raf-1 can apparently phosphorylate p53 under some circumstances (Jamal and Ziff, 1995), it is conceivable that BAG-1 interferes with p53's suppression of cell proliferation through effects on Raf-1 as well on Siah-family proteins. Given that BAG-1 did not prevent p53-mediated transactivation of a reporter gene, or alter p53 protein levels or p53 entry into the nucleus, we do not favor the hypothesis that BAG-1/Raf-1 interactions explain the ability of BAG-1 to suppress p53-induced proliferation.

Regardless of the mechanism by which BAG-1 interferes with p53 function, we speculate that *BAG-1* represents a novel type of oncogene which has multiple ways of promoting cell growth, including enhancement of the functions of Bcl-2, Raf-1, HGF-R and PDGF-R, as well as inhibition of Siah-1A. In the case of Raf-1, it has been shown that BAG-1 can bind to the catalytic domain of this kinase and stimulate increases in its kinase activity through a Ras-independent mechanism (Wang *et al.*, 1996). The interaction of BAG-1 with HGF- and PDGF-receptors has been correlated with enhanced generation of signals that selectively promote cell survival, apparently without influencing the signaling involved in mitogenesis (Bardelli *et al.*, 1996). Moreover, BAG-1 expression is inducibly increased by some growth factors, including IL-2, IL-3 and prolactin, in hematopoietic and lymphoid cells (Adachi *et al.*, 1996; Clevenger *et al.*, 1997), and gene transfer-mediated elevations in BAG-1 can abrogate growth factor dependence for growth and survival in some circumstances (Clevenger *et al.*, 1997). In addition, we have obtained evidence from immunohistochemical analysis of human tumors that BAG-1 expression is elevated in a significant proportion of breast and prostate adenocarcinomas, compared with normal mammary and prostate epithelial cells (manuscripts submitted). Taken together, these observations imply that BAG-1 is a potent cell growth regulator which alters the functions of several proteins involved in either cell proliferation or apoptosis, presumably through its effects on molecular chaperones.

Materials and methods

Plasmids

The cDNAs encoding various fragments of mouse BAG-1 were generated by PCR from the plasmid pcDNA3–mBAG-1 (Takayama *et al.*, 1995) using the following forward (F) and reverse (R) primers containing *Eco*RI and *Xho*I sites: BAG-1 (1–219), 5′-ACCGAATCCATGGCCAAGACCG-3′ (F1) and 5′-GCCACTCTCGAGTGCACTTCATTCA-3′ (R1); BAG-1 (1–172), F1 and 5′-ATGGTTGCGAATTCATGTTAATTGGTG-3′ (R2); BAG-1 (90–219), 5′-TGTTATGGTAGGACCATTGTGTC-3′ (F2) and R1; and BAG-1 (90–172), F2 and R2. The PCR products were digested with *Eco*RI and *Xho*I, then directly subcloned into the *Eco*RI and *Xho*I sites of the yeast two-hybrid vectors pGilda and pJG4-5 (gifts of E.Golemis and D.Buckholtz), which produces fusion proteins with a LexA DNA-binding domain or a B42 transactivation domain, respectively, at the N-terminus under the control of a GAL1 promoter (Estojak *et al.*, 1995). Alternatively, the cDNAs were subcloned into pGEX4T-1 in-frame with GST for expression in bacteria (Hanada *et al.*, 1995).

The cDNAs encoding various fragments of Siah-1A were generated by PCR from the plasmid pJG4-5 (clone #21) using the following forward (F) and reverse (R) primers containing *Eco*RI and *Xho*I sites: Siah-1A (97–298), 5′-GGAATTCTCCATTCGCAACTTGGCTA-3′ (F3) and 5′-CCGCTCGAGTCAACACATGGAAATAGTTACATT-3′ (R3); Siah-1A (46–102), 5′-GGGAATTCGCATCCAACAATGACTTGG-3′ (F4) and 5′-CCCCTCGAGCTAAGCCAAGTTGCGAATGGA-3′ (R4); Siah-1A (22–193), 5′-GGAATTCGCTACAGCATTACCTACCGG-3′ (F5) and 5′-CGCCTCGAGTCAGTCAACAGCACCAGGAAG 3′ (R5). The PCR products were digested with *Eco*RI and *Xho*I, then directly subcloned into the *Eco*RI and *Xho*I sites of the pJG4-5. The additional yeast vectors pEG202–Bax, pEG202–Fas, pEG202–HaRas(V12), pEG202–Lamin-C and pJG4-5–HaRas(V12) have been described previously (Sato *et al.*, 1994, 1995; Hanada *et al.*, 1995).

Epitope tag constructions were made by N-terminal addition of an oligonucleotide encoding the sequences MDYKDDDDK (FLAG epitope-tag) in the pCI vector (thus creating pCI–FLAG) and C-terminal addition of three tandem copies of the HA tag in the pcDNA3 vector (thus creating pcDNA3–3′-HA). The pJG4-5–Siah-1A (22–298), pGilda–BAG-1 (1–219) and pGilda–BAG-1 (1–172) plasmids were digested with *Eco*RI and *Xho*I and subcloned in-frame into the *Eco*RI and *Sal*I sites of pCI–FLAG. To create the Siah-HA-tag construct, PCR-amplification was performed using pCI–Siah (1–298) as a template and the primers, 5′-GGGAATTCGGACTTATGGCATGTAAACA-3′ (F6) containing a *Eco*RI site and 5′-GGAATTCGCTACAGCATTACCTACCGG-3′ (R5) containing a *Xho*I site. After digestion with *Eco*RI and *Xho*I, the Siah cDNA was subcloned into the pcDNA3-3′-HA vector. pCMV–p53wt and pCMV–$p53_{179}$ have been described previously (Unger *et al.*, 1992).

Yeast two-hybrid assays

Library screening by the yeast two-hybrid method was performed as described (Durfee *et al.*, 1993; Sato *et al.*, 1995a) using the pGilda plasmid encoding mouse BAG-1 as a bait, a human Jurkat T-cell cDNA library (gift from Brian Seed) and the EGY48 strain *Saccharomyces cerevisiae* (MATα, trp1, ura3, his, leu2::plexApo6–leu2). Cells were grown in either YPD medium with 1% yeast extract, 2% polypeptone and 2% glucose, or in Burkholder's minimal medium (BMM) fortified with appropriate amino acids as described previously (Sato *et al.*, 1994). Transformations were performed by a LiCl method using 0.25 mg of pJG4-5 cDNA library DNA and 5 mg of denatured salmon sperm cDNA. Clones that formed on Leu-deficient BMM plates containing 2% galactose or 1% raffinose were transferred to BMM plates containing leucine and 2% glucose, and filter assays were performed for β-galactosidase measurements as previously described (Sato *et al.*, 1994; Hanada *et al.*, 1995). From an initial screen of $\sim 1.6 \times 10^7$ transformants, 298 clones were identified which transactivated the *LEU2* reporter gene, based on ability to grow on leucine-deficient media. Of those, 30 colonies were also positive for β-galactosidase. These 30 candidate transformants were then cured of the LexA/BAG-1 bait plasmid by growth in media containing histidine and then mated with each of five different indicator strains of cells containing one of following LexA bait proteins: BAG-1 (1–219), Bax (1–171), v-Ras, Fas (191–335), or Lamin-C. The mating strain was RFY206 (MATa, his3Δ200, leu2–3, lys2Δ201, ura3–52, trp1Δ::hisG), which had been transformed with pGilda–BAG-1 or various control proteins and selected on histidine-deficient media. This resulted in 23 clones which displayed specific two-hybrid interactions with BAG-1. DNA sequencing analysis revealed four cDNAs encoding portions of Siah-1A.

Isolation of full-length human Siah-1A cDNAs

To obtain the complete sequence of human Siah-1A, cDNA fragments containing the 5′ end of human Siah-1A were PCR-amplified from Jurkat random primer cDNAs by using a forward primer 5′-GGGAATTCGGACTTATGGCATGTAAACA-3′ (F6) containing an *Eco*RI site and a reverse primer 5′-TAGCCAAGTTGCGAATGGA-3′ (R7), based on sequences of EST database clones (NCBI identification numbers: 159149 and 132546). The PCR products were digested with *Eco*RI and *Bam*HI, then directly subcloned into the *Eco*RI and *Sal*I sites of pcI plasmid into

which the cDNA derived from pJG4-5–Siah (22–298) had previously been cloned, as a *Bam*HI–*Xho*I fragment. The additional 5′ sequence information contained in the overlapping EST clones included a potential ATG start codon within the context of a Kozak consensus sequence. Stop codons are found in all three reading frames within the predicted 5′-untranslated region proceeding the open reading frame.

Transient transfection and cell proliferation assays

Human embryonic kidney 293 cells and human fibroblast GM701 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal calf serum (FCS), 1 mM L-glutamine and antibiotics. Cells (~5×10^5) in 60 mm plates were transfected with a total of 10 μg of plasmid DNAs by a calcium phosphate precipitation technique (Okayama *et al.*, 1987), which included 1 μg of pCMV–β-gal as a marker. After 48 h, the cells were harvested and the number of both viable and dead cells were evaluated by trypan blue dye exclusion assays. Efficiency of transient transfection was estimated by *in situ* β-galactosidase assay using a portion of the transfected cells (Zha *et al.*, 1996). The transient transfection efficiency of 293 and GM701 cells was consistently ⩾90% and ⩾50%, respectively.

Cells undergoing DNA synthesis were identified by addition of ^{3}H-thymidine (specific activity ~6.7 Ci/mmol; Amersham, Inc.) or BrdU (Boehringer Mannheim, Inc.) to culture medium. GM701 cells (~5×10^5) in 60 mm plates were transfected with 10 μg of plasmid DNAs. After 24 h, cells were harvested by detaching with Trypsin–EDTA solution (Irvine Scientific), seeded into 24-well plates at 10^5/ml cells, and either 10 μCi/ml ^{3}H-thymidine or 10 μM BrdU was added. Cultures were continued for 24 h to increase the yield of labeled cells compared with that obtained with shorter pulse-labelings. The ^{3}H-thymidine-labeled cells were harvested by rinsing cell monolayers with ice-cold phosphate-buffered saline (PBS) and applying 1 ml of 10% TCA. Precipitated DNA was recovered by centrifugation at 16 000 *g* for 10 min, washed with 5% of TCA three times and solubilized in 0.5 ml of 0.5 M NaOH. After neutralized by addition of 0.25 ml of 0.1 N HCl, DNA incorporated radioactivity was determined by scintillation counting. BrdU-labeled cells were fixed in 70% ethanol containing 20 mM glycine (pH 2.0) for 20 min at –20°C. Incorporated BrdU was detected by an immunofluorescence method involving incubation for 30 min with 6 μg/ml of a mouse anti-BrdU primary antibody (Boehringer Mannheim), followed by 1:50 (v/v) of FITC-labeled goat anti-mouse immunoglobulin (DAKO). The proportion of positive nuclei was assessed based on analysis of >1000 cells.

Immunofluorescence microscopy

GM701 cells (~5×10^5) in 60 mm plates were transiently transfected with 5 μg pCI–FLAG-Siah or 5 μg pCI–FLAG-BAG-1 with or without 5 μg pCI–Siah. After 24 h, cells were recovered by trypsinization, and seeded into fibronectin-coated 8-well chambers (Nalge-Nunc International). After culturing for an additional 24 h, the cells were fixed with 3.7% formaldehyde in PBS for 10 min, rinsed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 2 min and blocked with a solution of 3% BSA in PBS for 1 h. The cells were incubated with 10 μg/ml of mouse anti-FLAG M2 antibody (1:300; Kodak) for 1 h. The slides were washed for 5 min in PBS and then incubated with a 1:50 (v/v) of FITC-conjugated goat anti-mouse immunoglobulin (Dako) for 1 h. The washing step was repeated, before applying coverslips using Vectashield® mounting medium (Vector Laboratories, Inc.).

In vitro protein interaction assays

Siah-1A and BAG-1 mutant cDNAs in pGEX-4T-1 were expressed in XL-1-blue cells (Stratagene, Inc.), and affinity-purified using glutathione–Sepharose, as described (Takayama *et al.*, 1995). Purified GST fusion proteins (0.5–1.0 μg immobilized on 10–20 μl of glutathione beads) and 2.5 μl of rat reticulocyte lysates (TNT-Lysates; Promega, Inc.) containing ^{35}S-labeled, *in vitro* translated (IVT) proteins were incubated in 0.1 ml of HKMEN (10 mM HEPES pH 7.2, 142 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.1% NP-40) at 4°C for 30 min. The beads were washed three times with 1 ml HKMEN solution, followed by boiling in 25 μl of Laemmli–SDS sample buffer. The eluted proteins were analyzed by SDS–PAGE (12%) and detected by fluorography. Use of equivalent amounts of intact GST fusion proteins and successful IVT of all proteins were confirmed by SDS–PAGE analysis using Coomassie staining or autoradiography, respectively (data not shown).

Immunoprecipitation assays

293 cells (~2×10^6) in 100 mm plates were transiently transfected with 10 μg of pCI–FLAG-BAG-1 and 10 μg of pcDNA3–HA-Siah. After 48 h, cells were disrupted by sonication in 1 ml of HKMEN solution containing 0.2% NP-40, 0.1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin. After pre-clearing with normal mouse IgG and 10 μl protein A-agarose, immunoprecipitations were performed using 10 μl of anti-FLAG antibody M2-conjugated agarose (Sigma) at 4°C for 4 h. After extensive washing in HKMEN solution, immune complexes were analyzed by SDS–PAGE or immunoblotting using anti-HA antibody 12CA5 (Boehringer Mannheim), followed by HRPase-conjugated goat anti mouse immunoglobulin (Amersham, Inc.), and detection using an enhanced chemiluminescence (ECL) system (Amersham, Inc.).

p53 assays

Levels of p53 protein were assessed by immunoblotting using 0.4 μg/ml of either the anti-p53 monoclonal antibody Ab-6 for cell lines that contain wild-type p53 or a mixture of Ab-1 and Ab-2 (Oncogene Sciences, Inc.) for tumor lines containing mutant p53. Transient transfection reporter gene assays were performed using a Bax–luciferase plasmid which contains a p53-RE, essentially as described (Miyashita and Reed, 1995). Cells (2×10^5) were co-transfected with 0.4 μg of pGL3-Bax and 0.2 μg of pCMV–β-gal, in combination with 0.4 μg of either pCMV–p53 or pCMV–p53(179). After 48 h, cells were lysed and the relative amount of luciferase activity was measured according to the manufacturer's instructions (Promega), normalizing all values with respect to β-galactosidase activity.

RNase protection assay

A *Eco*RI–*Bam*HI fragment of the human Siah-1A cDNA was subcloned into pGEM1, digested with *Eco*RI and *in vitro* transcribed in the presence of [^{32}P]UTP with SP6 RNA polymerase to generate a probe for RNase protection assay measurements of Siah-1A mRNA levels. A 218 bp fragment of the human keratin 18 cDNA was used to generate a probe which served as an internal control. Total cellular RNA was prepared from 293 cells and 20 μg was incubated with 2×10^5 c.p.m. of ^{32}P-Siah-1A probe in hybridization buffer containing 40 mM PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA and 80% formamide for 16 h at 43°C. Samples were then treated with 1000 units of RNase A (Sigma) and T_1RNase (Sigma) for 40 min at 30°C, followed by analysis by electrophoresis in 8 M urea/5% polyacrylamide gels and autoradiography.

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