

Bag1 is a regulator and marker of neuronal differentiation

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Abstract

Bag 1 acts as a co-chaperone for Hsp70/Hsc70. We report here that stable over-expression of Bag1 in immortalized neuronal CSM14.1 cells prevents death following serum deprivation. Bag1 over-expression slowed the proliferative rate of CSM14.1 cells, resulted in increased levels of phospho-MAP kinases and accelerated neuronal differentiation. Immunocytochemistry revealed mostly nuclear localization of Bag1 protein in these cells. However, during differentiation *in vitro*, Bag1 protein shifted from predominantly nuclear to mostly cytosolic in CSM14.1 cells. To explore *in vivo* parallels of these findings, we investigated Bag1 expression in the developing mouse nervous system using immunohistochemical methods. Early in brain development, Bag1 was found in nuclei of neuronal precursor cells, whereas cytosolic Bag1 staining was observed mainly after completion of neuronal precursor migration and differentiation. Taken together, these findings raise the possibility that the Bag1 protein is expressed early in neurogenesis *in vivo* and is capable of modulating neuronal cell survival and differentiation at least in part from a nuclear location.

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Keywords: neuronal differentiation; Bag1; CSM 14.1 cells; mouse brain development; apoptosis; neuroprotection

Abbreviations: Bag1, Bcl-2-associated athanogene-1; NSE, neuron-specific enolase; MAPK, mitogen-activated protein kinase

Introduction

Bag1 (Bcl-2-associated athanogene-1) is the first identified member of a family of Hsp70-binding proteins containing a conserved C-terminal region termed the 'Bag domain'.^{1–3} The Bag domain binds tightly to the ATPase domain of the Hsp70 family of molecular chaperones and regulates their activity. Diversity in the N-terminal regions of BAG-family proteins permits their association with specific target proteins or targeting to subcellular locations.

Bag1 was identified by virtue of its ability to bind and collaborate with Bcl-2 in suppressing cell death.¹ Since then, multiple functions have been reported for Bag1, including interactions with the serine/threonine-specific protein kinase Raf, some tyrosine kinase growth factor receptors, and several steroid hormone receptors.^{4–6} At the cellular level, over-expression of Bag1 can result in various phenotypes, including enhanced tumor cell proliferation, promotion of cell motility and metastasis, and increased resistance to apoptosis.^{1,2,5,7–14}

Programmed cell death or apoptosis is a crucial feature ensuring proper development and differentiation of the nervous system. Moreover, in response to many injuries, such as stroke, neurodegenerative disease and trauma, differentiated neurons are known to induce the expression of stress proteins, including heat shock proteins.^{15,16} Bag1 is expressed in the developing and mature mammalian nervous system.^{17,18} However, detailed information about the expression and function of Bag1 during neuronal development, differentiation and death is currently limited. For example, Bag1 has been shown to prevent apoptosis in PC12 cells *in vitro* when over-expressed together with Bcl-2.⁹ A similar synergistic effect of Bag1 and Bcl-2 has been demonstrated in an *in vivo* model of photoreceptor cell death involving transgenic mice expressing these genes under the control of the rod opsin promoter.¹⁹ Furthermore, loss of Bag1 immunoreactivity in the rat brain after transient middle cerebral artery occlusion²⁰ and in olfactory neurons following synaptic target ablation²¹ suggests a potential role for Bag1 in suppression of neuronal death.

Two isoforms of the mouse Bag1 protein resulting from alternative translation initiation codon selection within a single mRNA have been reported.²² In contrast to the human Bag1 protein, little is known about the intracellular location of these two proteins. In the present study, we demonstrate that when stably over-expressed in the immortalized neuronal cell line CSM14.1, the short p29 form of mouse Bag1 resides predominantly in the nucleus. Bag1 not only prevents neuronal death but also promoted neuronal differentiation *in vitro* in these cells. During differentiation, Bag1 protein accumulated in the cytosol of CSM14.1 cells, becoming absent from the nucleus. An analysis of Bag1 expression during neuronal development in the mouse *in vivo* revealed similar patterns of Bag1 protein expression and intracellular location when compared to neuronal differentiation *in vitro*. The findings suggest that Bag1 is capable of modulating neuronal death and differentiation from a nuclear location.

Results

Over-expression of mBag1 in neuronal CSM14.1 cells

To over-express Bag1 in neuronal cells, we transfected CSM14.1 neuronal cells²³ with a plasmid containing flag-

tagged mouse p29 Bag1 driven by the neuron-specific enolase (NSE) promoter (Figure 1A). In a co-transfection, we introduced pBabePURO containing a puromycin resistance gene as selection marker. The plasmid containing a NSE-driven luciferase gene served as control vector. Prior to and after selection of single cell clones, gene expression was checked by immunoblot analysis employing antibodies against Bag1 (Bur 1680 and 1735) and Flag. Flag-mBag1, which migrates in gels at a slightly higher molecular weight than endogenous Bag1, was strongly expressed (Figure 1B). Out of 30 clones tested, 12 were positive for over-expression of Bag1. Two clones (#15 and 20) were chosen for further analysis.

Localization of Bag1 protein in CSM14.1 cells

To explore the intracellular location of Bag1 in CSM14.1 neuronal cells, we performed immunofluorescence microscopy analysis of our stably-transfected cells, employing two antibodies raised against different epitopes of Bag1. Compared to the levels of endogenous Bag1 expression in CSM14.1 cells, we observed a striking increase in nuclear immunofluorescence in cells over-expressing Bag1 (Figure 1C). In contrast, staining with preimmune serum and rabbit IgG produced no immunofluorescence, demonstrating the specificity of these results (not shown).

To verify our findings, we applied an antibody against Flag. As shown in Figure 1C, the tagged version of Bag1 was detected mainly in the nucleus of Bag1-transfected cells. In contrast, no immunofluorescence was observed in control-transfected CSM14.1 cells using anti-Flag antibody (Figure 1C).

Bag1 inhibits cell death after serum starvation

Serum starvation induces death of CSM14.1 cells maintained at 39°C.²³ After 2 days without serum, approximately 40% of wild-type cells were dead (Figure 2). This death can be largely attributed to apoptosis, since cells displayed fragmented nuclei (as visualized by DAPI staining) and could be protected by treatment with the broad-spectrum caspase inhibitor z-VAD-fmk (data not shown). As shown in Figure 2, stable Bag1 over-expression significantly prevented cell death measured at 24 and 48 h after serum deprivation ($P < 0.01$), while transfection with control vector (CSMluc) did not. Both of the stably-transfected clones tested, Bag15 and Bag20, which express the transgene at comparable levels (see insert in Figure 2), displayed substantially reduced cell death rates (~50%) when compared to control-transfected cells.

Since cell death prevention by stable over-expression of Bcl-2 in CSM14.1 cells has been reported previously,²³ we also compared Bag1 transfectants to Bcl-2 over-expressing CSM14.1 cells. As shown, Bcl-2 proved to be more effective than Bag1, abolishing cell death following serum deprivation almost completely (Figure 2).

Bag1 induces differentiation of CSM14.1 cells

We observed changes in morphology of Bag1 overexpressing cells even when cultured at the 'permissive' temperature of 32°C. In contrast to the small and round shape of wild-type cells, Bag1 over-expressing cells look larger and display a more polarized shape (not shown, compare Figure 1C).

Since differentiation of CSM14.1 cells is known to occur after switching temperature to 39°C,²³ we suspected that

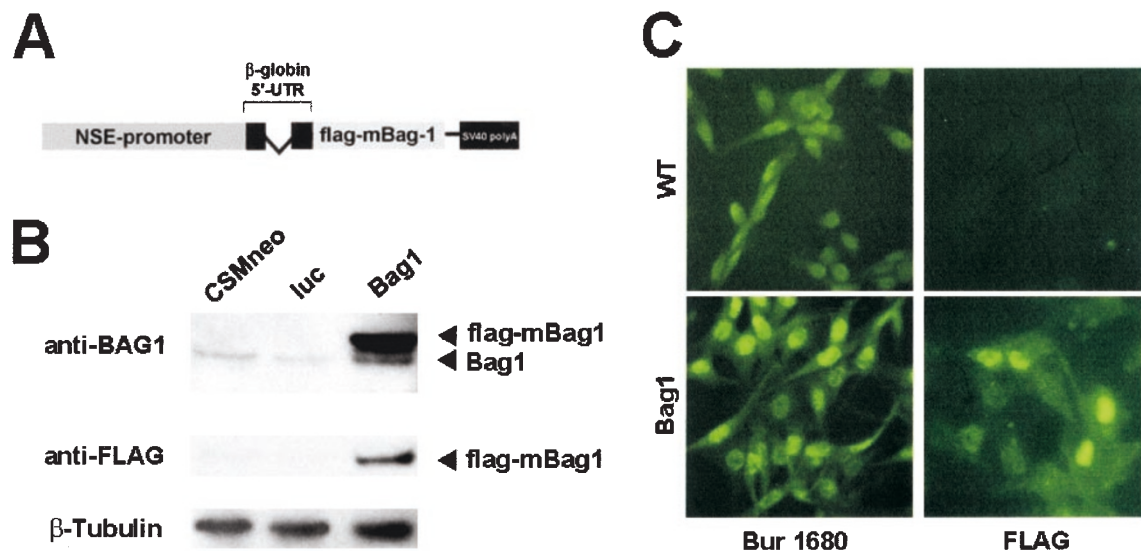


Figure 1 Generation of Bag1 over-expressing cells. (A) Cells were stably transfected with a plasmid containing the flag-tagged sequence of mouse Bag1 under control of the neuron-specific enolase promoter (NSE). (B) Immunoblot analysis of lysates (20 μg per lane) from wild-type anti-FLAG (CSMneo), empty vector-transfected (luc) and Bag1-transfected (Bag1) CSM14.1 cells prior to isolating clones, developed with anti-Bag1 antiserum Bur 1680 and visualized by ECL reagent on nitrocellulose membranes. The plasmid-derived flag-mBag1 and endogenous Bag1 are indicated by arrowheads. (C) Immunofluorescence microscopy was performed employing polyclonal Bag1 antiserum or monoclonal anti-FLAG antibody, followed by FITC-labeled secondary antibody (400 × magnification). Upper row shows control-transfected cells (WT), revealing nuclear and cytoplasmic expression of endogenous Bag1. No signal was detected with FLAG antibody. In contrast, Bag1 immunofluorescence in stable transfectants (lower row, Bag1) is strongly increased with predominantly nuclear localization of flag-mBag1 revealed by staining with the anti-FLAG antibody

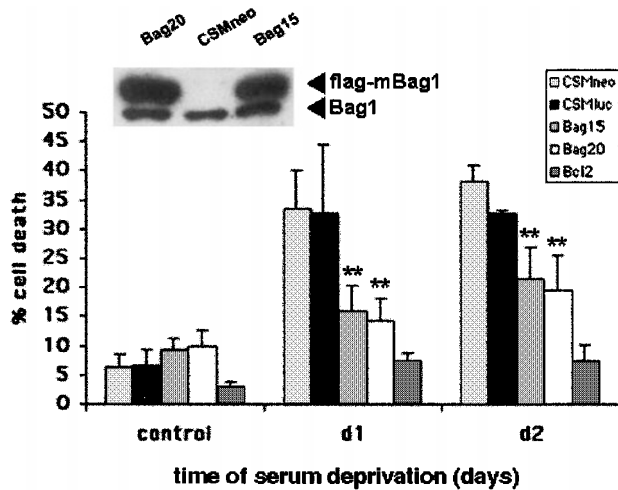


Figure 2 Bag1 is protective after serum deprivation. Cells were maintained at 'non-permissive' temperature for 2–3 days prior to serum-deprivation. Cell death was assessed by Trypan Blue exclusion assay (mean \pm s.d.; $n=6$). Two clones (15 and 20) expressing comparable levels of flag-mBag1 were analyzed (see insert). **significant when compared to CSM14.1 ($P < 0.01$)

Bag1 might promote differentiation of these cells. Since neuronal differentiation is associated with a cessation of cell proliferation, we first assessed the doubling time of control and Bag1 over-expressing CSM14.1 cells. We found a highly significant difference in the generation times of the respective cell lines at 32°C ($P < 0.01$). Cells over-expressing Bag1 were less proliferative, with an average generation time of 42 h, whereas wild-type CSM14.1 and empty vector transfected cells doubled on average every 25 h (Figure 3A).

To further explore the effects of Bag1 on differentiation of CSM14.1 cells, experiments were performed in which CSM14.1 cells were switched to non-permissive temperature of 39°C (where the SV40 large T-antigen is inactive) and cell morphology was compared at various times thereafter. Figure 3B, for example, contrasts the morphology of wild-type and Bag1 over-expressing cells at two different times after switching to 39°C. After 8 days at 39°C, most wild-type and control-transfected CSM14.1 cells had an enlarged cell soma containing a big nucleus. However, few of these cells had started to extend axon-like processes. In contrast, in cultures of Bag1 over-expressing cells, almost all of the cells were much smaller in size and assumed a more polarized shape. Virtually all of these cells had grown processes, which often aligned, forming bundles. Measurement of the length of these processes after 8 days at non-permissive temperature revealed that axon-like structures in Bag1 over-expressing cells had grown almost three times longer than processes in wild-type cells ($P < 0.01$; Figure 3C). After extended periods at 39°C, the morphological differences became even more pronounced. After 21 days at non-permissive temperature, Bag1 over-expressing cells were characterized by striking arborization of processes and formation of dense nests of axon-like connections between cells. In contrast, cells in

cultures of wild-type or control-transfected cells had a less differentiated appearance, with residual large cells and far fewer cellular processes (Figure 3B).

To correlate these differences in morphological changes with markers of the neuronal phenotype, we investigated the expression of neuronal differentiation-associated proteins in these cell lines. After extended time at non-permissive temperature, weak Neurofilament-200 staining could be detected in cultures of either control- or Bag1 over-expressing CSM14.1 cells (data not shown). In contrast, positive staining for NeuN antigen (a marker of post-mitotic neuronal nuclei) was observed in most Bag1 over-expressing cells by day 21 at 39°C, while NeuN staining was very weak or completely absent in control CSM14.1 cells (Figure 3D).

Interestingly, during neuronal differentiation at non-permissive temperature, there was a loss of nuclear Bag1 expression. Figure 3E illustrates the localization of Bag1 in stably transfected CSM14.1 cells after 28 days at non-permissive temperature using polyclonal antiserum against Bag1 (Bur 1680). In contrast to the mainly nuclear location of flag-mBag1 at permissive temperature (compare Figure 1C), most Bag1-transfectants over-expressing cells display an exclusively cytosolic location of Bag1, as revealed by double-labeling with DAPI (Figure 3E). As before, staining with preimmune serum and control rabbit IgG served as negative controls (not shown).

Bag1 over-expression induces MAPK-pathway

Given the accelerated differentiation of Bag1 over-expressing cells after switch to non-permissive temperature we hypothesized that the activation of MAP kinases, known to be important players in neuronal differentiation, contributes to this phenotype. As shown in Figure 4, Bag1 over-expressing cells showed increased levels of phospho-Erk1/2 at permissive temperature of 32°C when compared to wild-type cells. These high levels of Erk1 and 2 phosphorylation were maintained after switch to 39°C. In wild-type cells, only a modest increase in Erk-phosphorylation could be detected following temperature switch, while the expression of non-phosphorylated Erks did not change significantly in both cell types over time. Interestingly, the temperature switch induced increased levels of Hsp70 with similar kinetics in both wild-type and Bag1 over-expressing cells (Figure 4).

Bag1 expression during development of the mouse nervous system

Bag1 expression in the developing mouse nervous system was studied using immunohistochemical methods. At the onset of neurulation (E7.5–8), neural folds in the cephalic region contained only trace levels of Bag1 immunostaining. A single layer of pseudostratified columnar epithelium, constituting the neuroepithelium of the primitive neural tube, exhibited barely detectable cytosolic Bag1 staining (Figure 5A). With the formation of the three primary brain vesicles at E8.5–9.5, Bag1 nuclear staining appeared and was located predominantly in the differentiating neuroblasts of the rhombencephalon and mesencephalon, and at lower levels

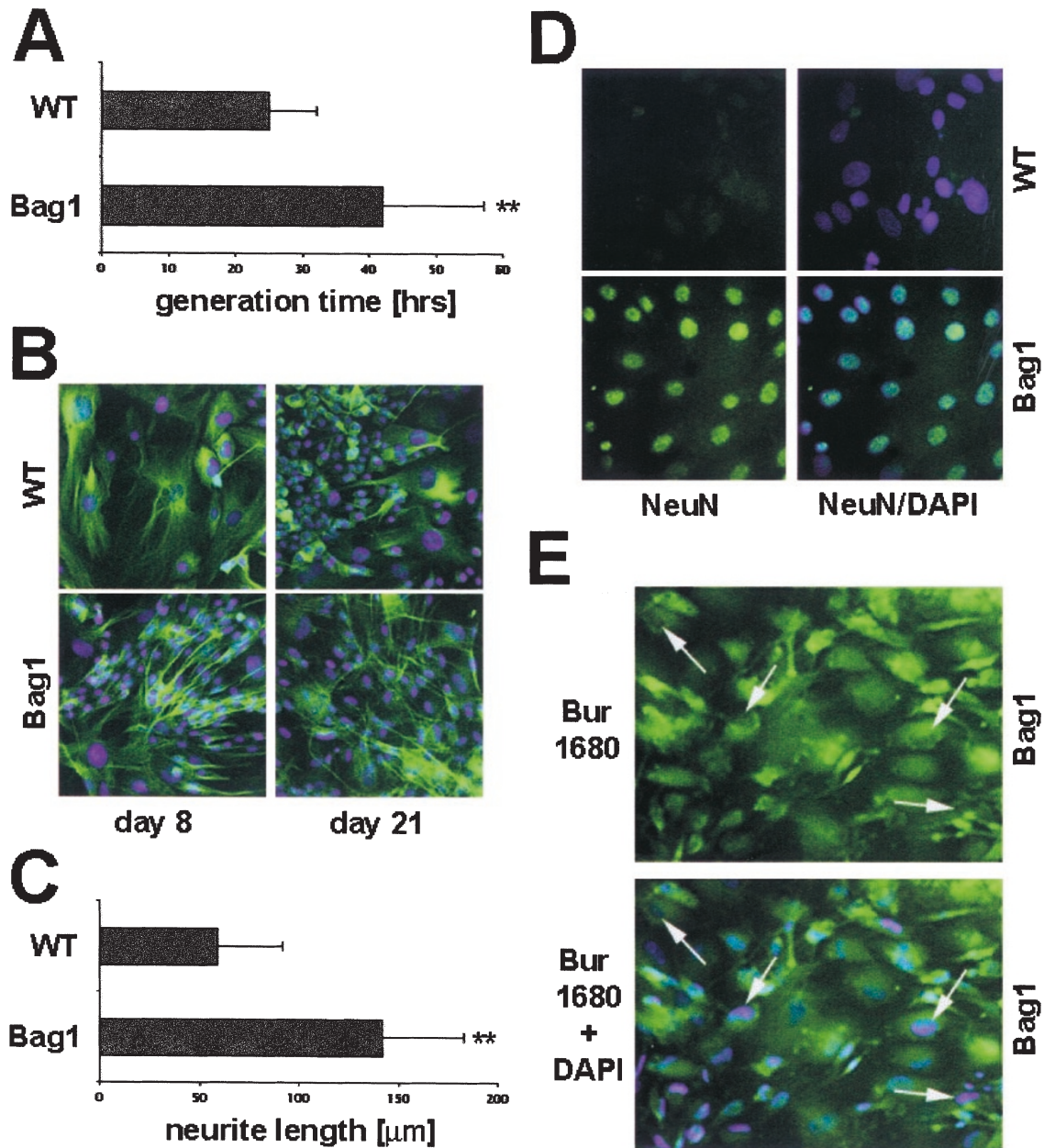


Figure 3 (A) Bag1 induces neuronal differentiation *in vitro*. Generation time was assessed by counting cell numbers during logarithmic growth at permissive temperature (32°C) over 1 week. Data were averaged for wild-type and control-transfected (WT) as well as two independent Bag1 over-expressing clones (Bag1). Average generation time (mean \pm s.d., $n=3$) was significantly higher in Bag1 over-expressing cells ($P<0.01$). (B) Immunostaining was performed using anti-Tubulin antibody (visualized with FITC-coupled secondary antibody). Cells were co-stained with DAPI. Photomicrographs show wild-type or control-transfected (upper row, WT), and Bag1 over-expressing cells (lower row, Bag1) on day 8 and 21 after switching to 'non-permissive' temperature (200 \times magnification). Note the morphologically advanced stage of Bag1 over-expressing cells with formation of dense nests of cellular processes on day 21. (C) Measurement of neurite extension in Bag1 over-expressing *versus* wild-type or control-transfected cells on day 8 after switch to 39°C. Data represent mean \pm s.d. ($n=75$ cells measured from three independent experiments). Processes are significantly longer in Bag1 over-expressing cells ($P<0.01$). (D) WT (upper row) and Bag1 over-expressing (lower row) cells were fixed after 28 days culture at 39°C and stained using FITC-labeled NeuN and DAPI (400 \times magnification). While NeuN staining in WT cells is absent or very faint, almost all nuclei in Bag1 over-expressing cells expressed the NeuN antigen. (E) Bag1 over-expressing cells (Bag1) were fixed after 28 days culture at 39°C and stained using polyclonal Bag1 antibody (Bur 1680, upper panel) followed by detection with FITC-anti-rabbit IgG. The same sample was stained with DAPI to reveal nuclei. As shown in the upper panel, Bag1 immunofluorescence is excluded from the nuclear region of differentiated CSM14.1 cells (arrows). Staining with DAPI confirmed that the Bag1-negative regions corresponded to nuclei (lower panel)

in the prosencephalon (Figure 5B,C). Numerous neuroblasts in the caudal part of the neural tube (which subsequently

differentiates into the spinal cord) contained moderate levels of Bag1 nuclear labeling (Figure 5D).

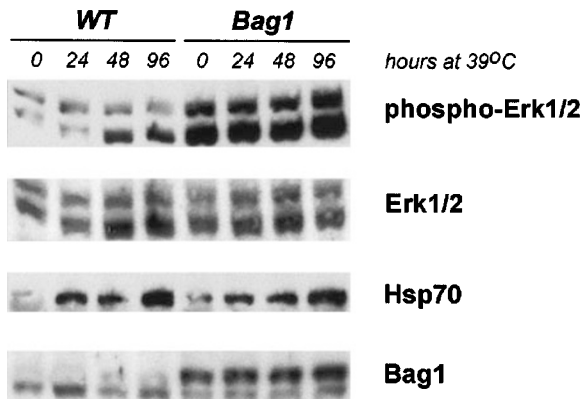


Figure 4 Bag1 over-expression activates MAPK pathway. Lysates of wild-type (WT) and Bag1 over-expressing (Bag1) cells at different time-points after switch to 39°C were subjected to SDS-PAGE and analyzed by immunoblot incubating the same membrane sequentially with phospho-Erk1/2, Hsp70, Bag1 and Erk1/2 antibodies. Bag1 over-expressing cells displayed markedly increased levels of phospho-Erk1/2

At a later stage of neural differentiation (E10–11), when the division of the primitive neural tube into three concentric layers occurs, neuroblasts in proliferative, periventricular matrix zone were consistently negative for Bag1. An obvious gradient of Bag1 expression appeared at this stage, associated with differentiation of the peripherally migrating progeny of ventricular neuroblasts to the intermediate mantle layer. The cells with early features of neuronal differentiation in the mantle layer, which gives rise to the gray matter of the central nervous system, demonstrated strong nuclear Bag1 immunostaining (Figure 5E).

At E12, when the various regions of the brain are more clearly defined, roughly half of the migrating postmitotic neurons evidenced not only intense nuclear staining but also increasing cytoplasmic Bag1 immunoreactivity (Figure 5F) in the hypothalamus, thalamus, and corpus striatum (the major derivatives of the diencephalon). At this time, a significant increase of Bag1 immunoreactivity also became apparent in the developing peripheral nervous system. Strong nuclear labeling was evident in the differentiating sensory neurons of the dorsal root ganglia (Figure 5G), and in the segmental ganglia along sympathetic trunks. The cranial ganglia, such as the facial (VII), acoustic (VIII), and the glossopharyngeal (IX) ganglion complexes revealed a similar distribution pattern (data not shown).

By E13–14 in the central nervous system of developing embryos, differentiating neuroblasts in the neopallial cortex (which is formed as cells from the mantle layer of the telencephalic vesicles migrate into the overlying marginal zone to constitute in due course the outer gray layer of the cerebral hemispheres) contained moderate levels of cytosolic Bag1, whereas the intensity of the nuclear signal had greatly declined. In the forming hippocampal plate, nuclear Bag1 immunoreactivity remained only in the rare residual migrating neuroblasts throughout most of the thickness of the plate (data not shown). This trend toward diminishing nuclear and increasing cytosolic Bag1 immunostaining in more differentiated neurons continued

throughout the remaining nervous system development in multiple regions of the brain and peripheral nervous system, including the spinal cord (Figure 5H,J), sympathetic trunk ganglia (Figure 5), olfactory bulb, pyramidal neurons of the CA3-CA4 sector of the hippocampus (Figure 5K,L), diencephalons, mesencephalon and rhombencephalon as well as the retina (Figure 5M,N). Control stainings performed with preimmune serum or using anti-Bag1 antibody that had been pre-adsorbed with Bag1 protein or peptide antigen confirmed the specificity of these results (Figure 5O, not shown).

During the later fetal life and into adulthood, selected types of neurons retained Bag1 expression, which was predominantly localized to the cytosol.

Discussion

Bag1 is an anti-apoptotic protein capable of binding to Bcl-2. Recently, binding of Bag1 to a variety of other intracellular proteins linking cell signaling, cell death and cellular stress responses has been shown.³ In the present report, we suggest a new function of Bag1 as a regulator of neuronal differentiation *in vitro* and as a marker of neuronal differentiation *in vivo* in the developing mouse nervous system.

For our *in vitro* studies, we used the neuronal cell line CSM14.1 which originated from rat nigral neurons immortalized by expression of temperature-sensitive SV40 large T antigen. These cells show a high proliferative rate at permissive temperature, while inactivation of large T antigen at non-permissive temperature induces neuronal differentiation.²³ Over-expression of Bcl-2 in these cells has been shown to protect them from a variety of death stimuli, including serum starvation.²³

To explore a potential role for Bag1 in the control of neuronal death, we stably over-expressed mouse Bag1 in CSM14.1 cells under the control of the neuron-specific NSE promoter.²⁴ Over-expression of Bag1 significantly reduced apoptotic cell death induced by serum deprivation in cultures of CSM14.1 cells. Similarly, Bag1 has been shown to reduce cell death following growth factor withdrawal in 3T3 fibroblasts, Ba/F hematopoietic cells and PC12 neural cells.^{1,7,9} In contrast, Roth and co-workers²⁵ reported synergistic effects of Bag1 and Bcl-2, but no cyto-protective effects of Bag1 alone in a model of glioma cell death following serum starvation.

Besides cyto-protective effects, Bag1 over-expression in CSM14.1 cells promoted neuronal differentiation. Previously, the protein kinase Raf-1 has been shown to play an important role in neuronal differentiation.²⁶ Bag1 interacts with Raf-1 and enhances its activity.^{6,27} The regions in Bag1 required for Raf-1-binding are overlapping with but distinct from those involved in Hsp70 binding.^{6,27} Raf kinase is a proximal element of the Ras-Raf-mitogen-activated protein kinases (MAPK) cascade. This pathway is a central effector of cellular differentiation during development²⁸ and seems also to subserve memory and synaptic plasticity in the post-mitotic, mature nervous system.²⁹ Consequently, Bag1 over-expressing neuronal cells displayed markedly increased levels of phospho-Erk1/2, the downstream kinases in the MAPK cascade. Interestingly,

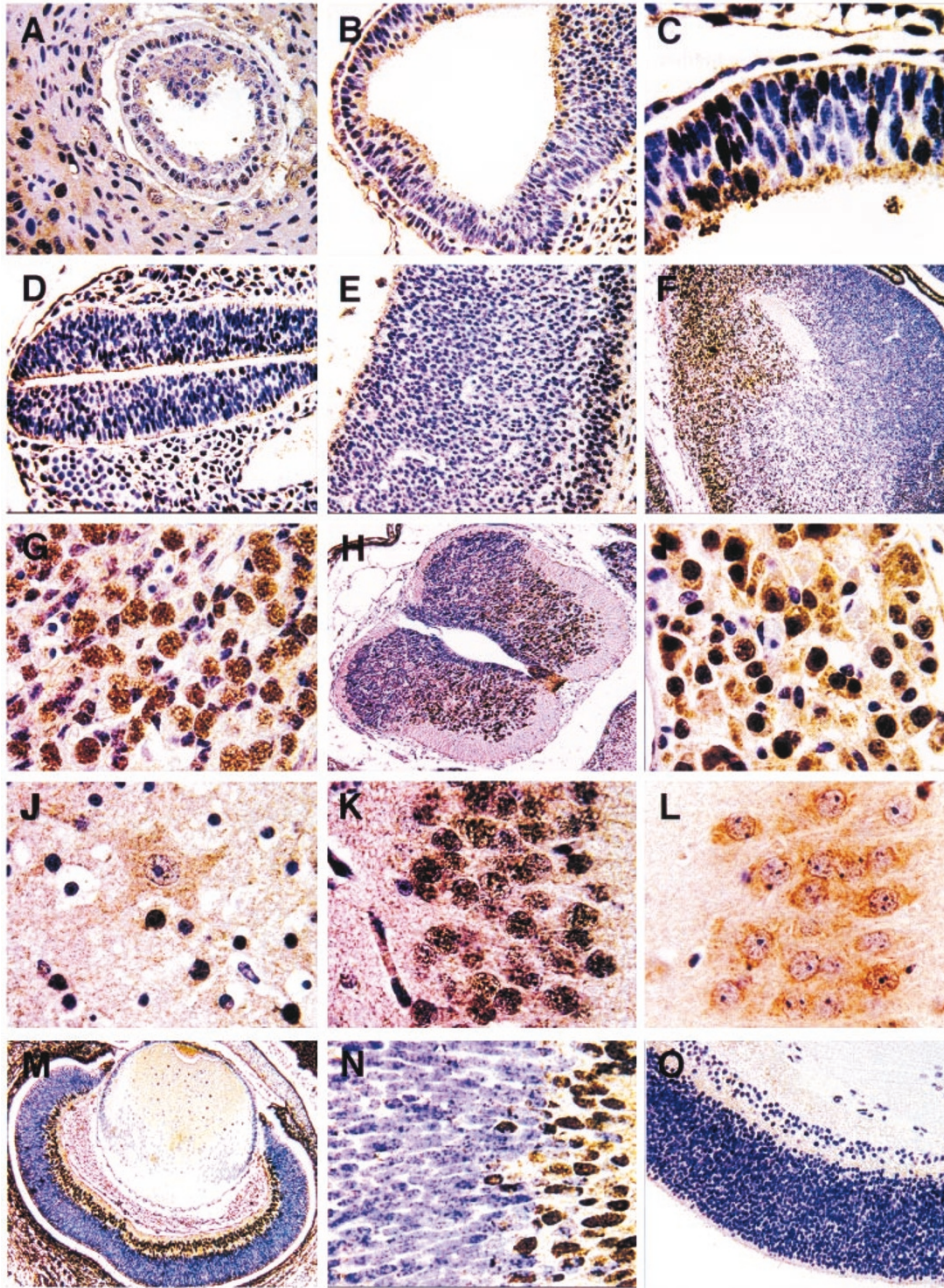


Figure 5 Bag1 expression in the developing nervous system of the mouse. Representative photomicrographs are shown for analysis of Bag1 expression in the mouse nervous system. Antibody detection was achieved using a DAB-based chromogenic method (brown) and nuclei were counterstained with hematoxylin. All results presented in this figure were obtained using a rabbit polyclonal antibody raised against a synthetic peptide (BUR1735), but similar results were observed using alternative antibodies (BUR1680 and 1702, not shown). (A) At the onset of neurulation (E7.5–8), the primitive neural tube exhibited barely detectable levels of cytosolic Bag1 (magnification 250 \times). (B,C) At E8.5, Bag1 nuclear immunostaining appeared in the differentiating neuroblasts of the primary brain vesicles (magnification 250 \times and 1000 \times , respectively). (D) At E8, neuroblasts in the caudal part of neural tube exhibit high levels of Bag1 nuclear expression (magnification 250 \times). (E) Only occasional neuroblasts in the proliferative, periventricular matrix zone show Bag1 staining, whereas the differentiating neuronal cells in the mantle layer demonstrate strong Bag1 immunoreactivity (E11) (magnification 250 \times). (F) The majority of neuronal cells in hypothalamus and basal part of corpus striatum demonstrate intense nuclear staining and increasing cytoplasmic signal for Bag1 (E12) (magnification 150 \times). (G) At E12, strong nuclear immunoreactivity is evident in the differentiating sensory neurons of the dorsal root ganglia (magnification 400 \times). (H) In the spinal cord (E14.5), note the elevated nuclear Bag1 immunoreactivity in the ventral motor neurons, and a negligible amount of this protein in the dorsal part (magnification 80 \times). (I) An appearance of cytosolic immunostaining for this protein is noticed in the sympathetic trunk ganglia (E17) (magnification 400 \times). (J) A terminally differentiated motor neuron in the spinal cord is shown, demonstrating loss of nuclear Bag1 staining at E17 (magnification 100 \times). High nuclear Bag1 expression in neurons of CA3 hippocampal sector in the later fetal stages (E17) (K; magnification 1000 \times), down-regulated in their adult counterparts (L; magnification 1000 \times). (M,N) At E17, the outer-most, proliferating layer of primitive neuroepithelial cells in the retina is mostly immunonegative. The neuroblasts in the inner layer, which will differentiate into the ganglion cells, contain high levels of nuclear Bag1 (magnification 100 \times and 1000 \times , respectively). (O) Control immunostaining using anti-Bag1 antiserum preadsorbed with 5 μ g/ml of synthetic peptide immunogen showed negative staining of the neural retina (magnification 250 \times)

increased levels of active MAP kinases were already present at permissive temperature and associated with altered morphology of the cells. However, expression of neuronal differentiation markers could only be observed after switch to non-permissive temperature of 39°C, where the large T antigen becomes inactive in these cells. It is tempting to speculate therefore that the signal for differentiation provided by MAPK activation was insufficient to overcome the proliferative stimulus of active SV40 large T antigen. However, after inactivating T antigen by switching to 39°C, increased levels of phosphorylated MAPKs in Bag1-expressing cells triggered differentiation into post-mitotic neurons.

We observed a significantly increased doubling time of Bag1-expressing CSM14.1 cells even at permissive temperature where large T antigen is active. Similarly, Bag1 was reported to slow glioma cell growth both in control and under serum-deprived conditions.²⁵ Recent findings support a role for Bag1 in cell growth regulation.^{13,27} Over-expression of Bag1, for example, can promote factor-independent growth of Interleukin 3-dependent lymphoid precursor cells⁷ and interfere with p53-mediated cell cycle arrest in epithelial tumor cell lines.¹³ In this regard, it has been shown that Bag1 binds to the human homologue of *Drosophila seven in absentia* (Siah),¹³ a p53-inducible gene.³⁰ Interestingly, in a transient transfection assay of the human kidney cell line 293T, over-expressed Siah-1 recruited Bag1 exclusively into the nucleus.¹³ Our data however argue that effects of Bag1 on cell proliferation are likely to be influenced by cellular background or lineage, given that Bag1 suppressed rather than stimulated growth of CSM14.1 neuronal cells. In line with this, Bag1 also failed to overcome p53-induced growth arrest in two human glioma cell lines.^{25,31}

The reasons for differences in the effects of Bag1 protein over-expression on proliferation of neural *versus* epithelial or hematopoietic lineage cells are unknown, but a possible clue may come from contrasting the intracellular locations of this protein in cells. In the mouse, two isoforms of Bag1 have been described, with the shorter one studied here representing the more abundant gene product by far.²² The intracellular location of this Bag1 protein isoform may vary depending on cell background, environment and stage of differentiation.^{22,32} In CSM14.1 cells, we detected Bag1 transgene expression predominantly in the nucleus of undifferentiated CSM14.1 cells. In contrast, p29 Bag1 is reportedly found predominantly in the cytosol of epithelial and hematopoietic cells.^{7,22} After shifting CSM14.1 cells to non-permissive temperature to allow differentiation, Bag1 protein accumulated in the cytosol over time, becoming absent from the nucleus. Accordingly, Bag1 presumably interacts with different molecular targets and exerts different effects depending on its intracellular location and cell type.

Interestingly, in the developing mouse nervous system, we found a similar situation with respect to Bag1 expression patterns, suggesting that Bag1 may modulate neuronal differentiation from a nuclear location. During neuronal differentiation in the mouse brain, Bag1 expression was highest in differentiating neuroblasts. Bag1 immunoreactivity was restricted to the nuclei of these cells,

whereas low levels of cytosolic Bag1 were detected later in development in mature neurons. This shift of Bag1 expression from predominantly nuclear to mostly cytosolic, in parallel with neuronal maturation, was observed in all parts of the nervous system.

In summary, our results suggest a new function for Bag1 as a marker and regulator of neuronal differentiation. Future studies investigating the association of Bag1 expression with neuronal differentiation *in vivo*, particularly the generation of Bag1 transgenic and knock-out mice, should yield additional insights which might eventually be exploited for neuronal preservation or replacements after stroke, neurodegeneration, or other diseases.

Materials and Methods

Plasmid constructions

A plasmid was constructed for expression of flag-mouseBag1 (mBag1) under control of the neuron-specific enolase (NSE) promoter. First the 1.8 kb 5'-fragment of the rat NSE-promoter,²⁴ kindly provided by G Sutcliffe, was subcloned into pBSKII using the *EcoRI* and *HindIII* restriction sites. In a second step, this plasmid was digested with *BamHI* and *HindIII*. The resulting NSE-promoter containing fragment was used to replace the HSV-TK promoter in pRL-TK (Promega) between the *BglII* and *HindIII* sites. The resulting construct containing a luciferase gene under the control of the NSE promoter served as control vector. Subsequently, flag-mBag1 was inserted at the *NheI* and *NotI* sites from pCI-flag-mBag1.¹

Stable transfections

The rat nigro-striatal cell line CSM14.1, immortalized by introduction of the temperature sensitive SV40 large T antigen, and a Bcl-2 transfectant CSM 14.1-Bcl-2²³ were kindly provided by Dale Bredesen. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 1 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate either in 32°C at permissive or 39°C at non-permissive temperature.²³ For stable transfection, 50–70% confluent CSM cells in 6-well plates were incubated with Gene Porter II according to the supplier's protocol (Gene Therapy Systems) in serum-free medium containing 3 µg of plasmid DNA representing a 5:1 ratio of specific plasmid:puromycin-resistance plasmid (pBabe-puro). After 3 h at 37°C, serum-containing medium was added and the cells were incubated overnight. Finally, the transfection agent was replaced by 10% serum-containing medium and the cells were transferred to 32°C. Selection with 4 µg puromycin in complete medium was started the next day. After 5 days, 0.5 cells per well were seeded in 96-well plates with selection medium, and 3 to 4 weeks later wells containing single clones were identified by light microscopy and the cells transferred to larger plates for expansion and further processing.

Cell death, cell division assays and measurement of neurite outgrowth

Cell death was induced by serum deprivation as published previously.²³ 10⁵ cells were plated in 6-well plates and maintained in 39°C. Prior to serum deprivation, cells were washed three times in serum-free medium. Cell death was assessed by Trypan Blue exclusion after 24 h, 2, 3, and 4 days in serum starvation. Experiments were repeated six times.

Doubling times were determined during logarithmic growth in 24-well plates. 10^4 cells were plated and daily cell counts were performed by Trypan Blue exclusion assay for 1 week.

The length of outgrowing neurites was assessed on day 8 at 39°C in three independent experiments on randomly picked cells using ImagePro-Plus software.

Statistics were performed using two-tailed Student's *t*-test.

Antibodies

Polyclonal antisera for Bag1 were generated in rabbits using synthetic peptides or GST-fusion protein immunogens. A peptide (NH₂-CNERVDLLVTPQQNSEPVVQD-amide) corresponding to residues 26–45 of the mouse Bag1 protein, was synthesized with an N-terminal cysteine appended to permit conjugation to maleimide-activated carrier proteins KLH and OVA (Pierce, Inc.), as described previously.³³ This peptide conjugate was used to generate a polyclonal antiserum (#1735) in rabbits.²² An additional anti-Bag1 serum (#1680) was generated in rabbit using a GST-mouse Bag1 (8–219) fusion protein.¹ The generation and characterization of a rabbit anti-mouse Bag1 antiserum targeted against amino acids 204–219 (#1702) have been described.¹

Immunoblot assays and immunofluorescence

Cell lysates were prepared at different times after switching cells to non-permissive temperature using RIPA buffer as described.³⁴ Proteins (20 µg per lane) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% skim milk, 2% bovine serum albumin (BSA) in TBST (10 mM Tris [pH 7.5]; 142 mM NaCl; 0.1% Tween-20) at room temperature for 2 h, blots were incubated in the same solution with various primary antibodies including polyclonal antisera against Bag1 (Bur 1735 and Bur 1680; 1:1000; see above), phospho-Erk1/2 and Erk1/2 (Cell Signaling; 1:1000), as well as monoclonal antibodies against Flag (Sigma M2 or M5; 3 µg/ml) and Hsp70 (ABT; 1:5000), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Biorad) secondary antibodies. Bound antibodies were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham).

For immunofluorescence, wild-type cells and those stably transfected with either empty vector or flag-mBag1 were trypsinized and seeded into chamber slides. Cells were either maintained in 32 or 39°C until further processing. After various lengths of time, cells were washed in PBS and fixed in PBS containing 4% paraformaldehyde for 5 min at room temperature, followed by several washing steps in PBS. Permeabilization was performed in 0.3% Triton X-100/PBS for 5 min with subsequent preblocking in PBS containing 2% normal goat serum. Cells were incubated in blocking solution containing the following primary antibodies: Bur 1680 and 1735 (1:100), anti-Tubulin (1:2000), anti-Flag M5 (1:250), NeuN (Sigma, 1:50). After washing three times in PBS and incubation with FITC-conjugated secondary anti-mouse or anti-rabbit antibody (Dako, 1:50) for 2 h at room temperature, slides were covered with Vectashield mounting medium with or without 1.5 µg/ml 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and sealed with Cytoseal 60 mounting medium (Stevens Scientific).

Immunohistochemical analysis of mouse embryos

The timing and distribution of Bag1 protein expression in the developing nervous system were assessed in paraffin sections derived from embryos and postnatal mice of the NMRI or FVB strains.

All procedures were approved by the institutional animal care committee. Prenatal development was studied on a closely spaced series of mouse embryos at daily intervals from 6 days of gestation (E6) to postnatal day 4 (P4). Twenty-five mice were studied at weekly intervals after that time until adulthood. Mice were mated overnight, and the morning the vaginal plug appeared was designated as embryonal day 0.5 (E0.5). The day of birth was termed as postnatal day zero (P0). All embryos were taken from mice which had been killed by over-dose of carbon dioxide. At E4–9, the uterus was excised and fixed with the embryos *in situ*. For the later embryos, each embryo was dissected from the uterus, freed from the extra-embryonic membranes and immediately placed in the fixatives, either Bouin's fixative, or zinc-buffered formalin (Z-fix; Anatech LTD, Battle Creek, MI, USA). Immersion time varied from 2 days for early stages to 5–7 days for fetal and postnatal specimens. Altogether, tissue specimens from 58 embryos and 29 mice after birth have been paraffin-embedded according to routine procedures.

Dewaxed tissue sections were exposed to polyclonal antibodies and confirmed to be specific for Bag1. The sections were immunostained using a diaminobenzidine (DAB)-based detection method as described in detail, employing either an avidin-biotin complex reagent (Vector Laboratories) or the Envision-Plus-Horse Radish Peroxidase (HRP) system (DAKO) using an automated immunostainer (Dako Universal Staining System).^{33,35} The dilutions of antisera typically employed were 1:3500 (v/v) for #1735, 1:5000 for #1680, and 1:2500 for #1702.

To verify specificity of the results, the immunostaining procedure was performed in parallel using preimmune serum or anti-Bag1 antiserum preadsorbed with 5–10 µg/ml of synthetic peptide immunogen.

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