Published in final edited form as:

J Cell Physiol. 2007 March ; 210(3): 676–683. doi:10.1002/jcp.20865.

Evidence for BAG3 Modulation of HIV-1 Gene Transcription

ALESSANDRA ROSATI^{1,2}, ARTURO LEONE¹, LUIS DEL VALLE², SHOHREH AMINI^{2,3},
KAMEL KHALILI^{2,*}, and MARIA CATERINA TURCO¹

¹*Department of Pharmaceutical Sciences (DiFarma), University of Salerno, Salerno, Italy*

²*Department of Neuroscience and Center for Neurovirology, Temple University School of Medicine, Philadelphia, Pennsylvania*

³*Department of Biology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania*

Abstract

A family of co-chaperone proteins that share the Bcl-2-associated athanogene (BAG) domain are involved in a number of cellular processes, including proliferation and apoptosis. Among these proteins, BAG3 has received increased attention due to its high levels in several disease models and ability to associate with Hsp70 and a number of other molecular partners. BAG3 expression is stimulated during cell response to stressful conditions, such as exposure to high temperature, heavy metals, and certain drugs. Here, we demonstrate that BAG3 expression is elevated upon HIV-1 infection of human lymphocytes and fetal microglial cells. Furthermore, BAG3 protein was detectable in the cytoplasm of reactive astrocytes in HIV-1-associated encephalopathy biopsies, suggesting that induction of BAG3 is part of the host cell response to viral infection. To assess the impact of BAG3 upregulation on HIV-1 gene expression, we performed transcription assays and demonstrated that BAG3 can suppress transcription of the HIV-1 long terminal repeat (LTR) in microglial cells. This activity was mapped to the κB motif of the HIV-1 LTR. Results from *in vitro* and *in vivo* binding assays revealed that BAG3 suppresses interaction of the p65 subunit of NF-κB with the κB DNA motif of the LTR. Results from binding and transcriptional assay identified the C-terminus of BAG3 as a potential domain involved in the observed inhibitory effect of BAG3 on p65 activity. These observations reveal a previously unrecognized cell response, that is, an increase in BAG3, elicited by HIV-1 infection, and may provide a new avenue for the suppression of HIV-1 gene expression.

INTRODUCTION

The neuropathological hallmarks of HIV-1 infection of the brain from AIDS patients exhibiting neurological problems are the presence of multinucleated giant cells, astrogliosis, microglial nodules, macrophage infiltration, white matter pallor, reduced synaptic density, and neuronal loss in the cortex and basal ganglia (Stout et al., 1998; Everall, 2000). In a significant subset of infected individuals this is associated with a syndrome of cognitive and motor abnormalities called HIV-1-associated dementia (HAD) (Albright et al., 2003; Trujillo et al., 2005). Results from histopathological studies demonstrated infection of microglia, macrophages, and to some limited extent, astrocytes by HIV-1 in the brains of AIDS patients with encephalopathy (Torres-Munoz et al., 2001; Trillo-Pazos et al., 2003; Jones and Power, 2006; Petito, 2004). A limited, if any, infection of neurons by HIV-1 has also been reported (Torres-Munoz et al., 2001; Trillo-Pazos et al., 2003; Jones and Power, 2006; Petito, 2004). Virus entry into the host cells usually

*Correspondence to: Kamel Khalili, 1900 North 12th Street, 015-96, Room 203, Philadelphia, PA 19122. E-mail: E-mail: kamel.khalili@temple.edu.

In fond memory of Professor Arturo Leone.

results in profound alterations in cellular gene expression leading to deviations in cell physiology and behavior. At the same time, cells may develop a defense mechanism that blocks or decreases production of viral RNAs and proteins, and hence, diminishes the infection cycle. These events may involve alterations in the morphology and physiology of the cells caused by dysregulation of several cellular genes responsible for the control of the cell cycle (Kundu et al., 1998), apoptosis and necrosis (Gabuzda and Wang, 2000; Jones and Power, 2006), formation of syncytia (Lifson et al., 1986), and many other biological events.

Expression of the HIV-1 genome is mediated through the long terminal repeat (LTR) sequence, which requires the participation of several virus-encoded regulatory proteins including the potent transactivator Tat (Cullen, 1990; Morris and Rossi, 2004; Trujillo et al., 2005). In the early stages of infection, Tat mediates transactivation of the HIV-1 LTR promoter through its interaction with the Transactivation response region (TAR) an RNA sequence located in the leader of the viral transcript. In addition to TAR, the LTR contains the core promoter sequence comprised of a TATA box and a GC-rich motif at the 3' end of a series of enhancer sequences. The κ B motif, which is the target for the binding of NF- κ B inducible factors, is one of the most extensively studied enhancer motifs that has an established functional role in viral gene expression (Mufson et al., 1992; Desai-Yajnik and Samuels, 1993; Perkins et al., 1993). Previous studies by several laboratories demonstrated that the interaction of NF- κ B subunits, such as p65 and p50, with the κ B motif is critical for basal and Tat-induced activation of the viral promoter (Nabel and Baltimore, 1987; Taylor and Khalili, 1994).

p65 is one of the most studied members of the NF- κ B family and its activation is induced by a broad range of biological and pharmacological agents (Kaltschmidt et al., 2005). The activation of p65-containing NF- κ B dimers results from the degradation of I κ B, a cytoplasmic inhibitor which, under unstimulated conditions, retains NF- κ B factors in the cytosolic compartment. Several cytokines and immunomodulators, as well as other stimuli, induce the phosphorylation and subsequent degradation of I κ B and thus cause nuclear entry of the NF- κ B complex where it stimulates responsive promoters such as the HIV-1 LTR. Earlier studies have identified several cellular and viral proteins that can associate either directly or indirectly with the p65 subunit of NF- κ B and alter its activities (Ouivy and Van Lint, 2004).

Co-chaperone proteins that share the Bcl-2-associated athanogene (BAG) domain are characterized by their interaction with heat shock proteins and other partners (steroid hormone receptors, Raf-1, and others) involved in regulating a number of cellular processes, including proliferation and apoptosis (Takayama et al., 1999; Doong et al., 2000, 2002; Takayama and Reed, 2001). One BAG family member is BAG3, also known as CAIR-1 or Bis (Lee et al., 1999; Antoku et al., 2001; Briknarova et al., 2001, 2002; Liao et al., 2001; Takayama and Reed, 2001; Doong et al., 2002, 2003; Chroboczek et al., 2003; Pagliuca et al., 2003; Romano et al., 2003a,b; Bonelli et al., 2004; Chen et al., 2004; Dong et al., 2005). BAG3 forms a complex with Hsp70 (Takayama et al., 1999), a protein that assists polypeptide folding, can mediate altered peptide delivery to proteasome (Young et al., 2003) and is able to modulate apoptosis by interfering with cytochrome c release, apoptosome assembly, and other events in the death process (Beere, 2005). In addition, BAG3 polypeptide binds to phospholipase C-gamma (Doong et al., 2000) and possibly other partners (Takayama and Reed, 2001; Doong et al., 2002). Notably, we observed that BAG3 expression can be induced by some stressful agents such as high temperatures or heavy metals, and possibly viral infection. Activation of BAG3, in turn, can influence several cellular processes that are required for normal behavior of cells (Pagliuca et al., 2003).

Here, we demonstrate that BAG3 levels are induced upon HIV-1 expression, and that BAG3 interacts with the p65 subunit of NF- κ B and that this interaction affects the ability of the HIV-1 LTR to be responsive to the NF- κ B pathway.

MATERIALS AND METHODS

Plasmids and siRNAs

BAG3 deletion mutants were created from the full-length cDNA derived from human brain RNAs and placed in the pcDNA 6.1 expression vector (Invitrogen, Grand Island, NY) using *EcoRI/XhoI* restriction sites. The resulting proteins were His-Myc tagged at the C-terminus. The forward primer used for amplification of cDNAs was 5'-ATTTGGAATTCAAAAATGAGCGCCGCCACCCAC-3'; the reverse primers were: BAG3 FL (BAG3 full-length protein): 5' ATT GAT CTC GAG AAC GGT GCT GCT GGGT-3'; BAG3 1-502: 5'-ATTGATCTCGAGACATCAATGGCTTTCTGT-3'; BAG3 1-418: 5'-ATTGATCTCGAGCCTGGATGTTTTGGGGGA-3'.

The HIV-1 LTR reporter plasmids -120/+66 expression and its deletion mutants, plasmids for p65 and Tat were previously described (Amini et al., 2002; Sweet et al., 2005; Chipytisina et al., 2006). A specific siRNA targeting BAG3 mRNA (5'-AAGGUUCAGACCAUCUUGGAA-3') and a scrambled siRNA (5'-CAGUCGCGUUUGCGACUGG-3') were purchased from Dharmacon, La Fayette, CO.

Cell cultures

Primary human fetal microglial cultures were prepared from 8- to 12-week-old human fetal brain tissue (purchased from Advanced Bioscience Resources, Inc., Alameda, CA) by a modified procedure based on the methods by Cole and Vellis (1997) and Yong and Antel (1997). The human cell lines U87MG and SupT1, derived from an astrocytoma and a lymphoblastic leukemia, respectively, and the human epithelial cell line HeLa, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). U87MG and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, while SupT1 cells were grown in RPMI with 10% fetal bovine serum. All the cell culture media and supplements were purchased from Gibco (Invitrogen).

Biopsies and immunohistochemistry

A total of 12 biopsies from patients with HIV-encephalopathy were collected from the Manhattan Brain Bank at Mount Sinai School of Medicine in New York, New York (kindly provided by Dr. S. Morgello). The tissue, which had been previously fixed in 10% buffered formalin, was embedded in paraffin and sectioned at 4- μ m thickness. Immunohistochemistry (IHC) was performed using the avidin-biotin peroxidase system, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Our modified protocol includes deparaffination in xylene, re-hydration in alcohol up to water, non-enzymatic antigen retrieval with citrate buffer, pH 6.0 heated to 95°C for 30 min, and endogenous peroxidase quenching. After rinsing with PBS and blocking with normal goat serum for 1 h at room temperature, the sections were incubated with a rabbit polyclonal anti-BAG3 antibody (1:500 dilution) overnight in a humidified chamber. Then sections were rinsed with PBS and incubated with a biotinylated anti-rabbit secondary antibody (1:200 dilution) and avidin biotin complexes (ABC Kit, Vector Laboratories) 1 h at room temperature. Finally, the sections were developed with diaminobenzidine (Sigma, St. Louis, MO), counterstained with hematoxylin, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). For double labeling immunofluorescence, after incubation with BAG3 antibody, sections were incubated with a FITC-tagged secondary antibody for 1 h and thoroughly washed with PBS. Then sections were blocked with normal goat serum and incubated overnight with a mouse monoclonal anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Finally, a second rhodamine-tagged anti-mouse secondary antibody was incubated for 1 h, sections were coverslipped with a mounting media (Vector Laboratories), and visualized with a UV light source fluorescence microscope (Nikon Eclipse TE300).

Transfections and luciferase assays

U87MG cells and human microglial cells were co-transfected with 2 µg of a plasmid containing (−120 to +66) LTR-driven firefly luciferase vector and 0.5 µg TK-driven Renilla luciferase plasmid using lipofectamine (Invitrogen). Cells were also transfected with combinations of 2.5 µg of CMV-p65, 1 µg of CMV-Tat, and 2.5 µg of CMV-BAG3 expressing plasmids. Luciferase activity was measured after 48 h with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Bar graphs depict the means of relative transcription activities ± standard deviations (duplicate determinations). U87MG cells were transfected with siRNAs with TRANSIT-TKO (Mirus, Madison, WI) at final concentration of 200 nM. Cells were collected after 72 h.

Western blot analysis and antibodies

Cells were harvested, washed twice with DPBS 1× solution (Mediatech, Inc., Herndon, VA). Total proteins were extracted on ice with TNN buffer (50 mM tris pH 7.5, 150 mM NaCl, 0.5% NP40) supplemented with a protease inhibitor cocktail (Sigma). The lysates were centrifuged at 14,000 rpm at 4°C and the soluble fractions were collected. Protein concentrations were measured using a Bio-Rad Protein Assay (BioRad Laboratories, Inc., Hercules, CA). Equal amounts of total protein (30 µg) from each sample were separated electrophoretically in a 12% SDS–PAGE and blotted onto a nitrocellulose membrane (Hybond-P; Amersham life Sciences Corp., St. Louis, MO). Detection of proteins was performed using a polyclonal antibody raised against the full-length recombinant BAG3 protein (Romano et al., 2003a,b), an anti-p65 polyclonal antibody (C-20, Santa Cruz Biotechnology, CA) or a polyclonal antibody (obtained through the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH) against HIV-1 Tat. An anti-α-tubulin monoclonal antibody (Sigma) or an anti-Grb2 monoclonal antibody (BD biosciences, San Diego, CA) were used as control to monitor equal loading conditions. Secondary antibodies were purchased from Pierce (Rockford, IL). Immunodetection was performed using the ECL plus kit (Amersham Biosciences, Piscataway, NJ) according to the protocol provided by the manufacturer.

Co-immunoprecipitation

U87MG cells were transfected with BAG3 FL constructs or with deletion mutants using lipofectamine (Invitrogen). Three-hundred micrograms of proteins were used for immunoprecipitation with 3 µg of Myc-Tag antibody (monoclonal antibody 9B11, Cell Signaling, Danvers, MA) and incubated at 4°C O.N., on a tube rotator. Twenty-five microliters of protein A sepharose (Amersham Biosciences, Piscataway, NJ) were then added and the immunocomplexes were precipitated and washed five times with TNN buffer. Proteins obtained from immunoprecipitations and 30 µg of total protein were loaded on a SDS–PAGE and blotted on a nitrocellulose membrane. BAG3-deleted forms were detected using a His C-term antibody (monoclonal antibody, Invitrogen), while an anti-p65 polyclonal antibody (C-20, Santa Cruz Biotechnology, CA) was used to detect NF-κB p65.

EMSA and ChIP assays

U87MG cells were transfected as described above. Nuclear extracts were obtained using NE-PER kit (Pierce) and incubated with a ³²P-labeled probe containing the nucleotide sequence of the HIV-1 LTR κB element (5'-AGTTGAGGGGACTTTCCCAGGC-3' and 5'-GCCTGGGAAAGTCCCCTCAACT-3'). DNA-protein complexes were separated on 6% DNA retardation polyacrylamide gel.

ChIP assay was performed as described by the manufacturer (Upstate Cell Signaling Solutions, Charlottesville, VA). Primers used for PCR were (−120) AACTGGTACCCCGAGAGCTGCA and (+66) TTGAGGATCCAGCAGTGGGTTTC. A

fraction from the eluted complexes was saved and run on a 10% SDS-PAGE to verify the presence of the transcription factor NF- κ B p65.

HIV-1 infection

SupT1 cells in the log phase of growth or human fetal microglial cells plated at 70% of confluency were infected with JR-FL of HIV (obtained from the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH). Cells were incubated with virus stock in a small volume of serum free media for 2 h at 37°C; the cells were then washed twice with PBS and new fresh medium containing 2% of FBS was added. Cells were harvested at indicated days and HIV-1 infection was assessed by light microscopy for the syncytia formation in SupT1 cells and by Western blot for HIV-1 Tat in human fetal microglial cells.

RESULTS AND DISCUSSION

Immunohistochemical studies, that were performed in a collection of well-characterized cases of HIV-encephalopathy utilizing a polyclonal antibody raised against the carboxy-terminal domain of BAG3, revealed a noticeable upregulation of BAG3 levels in cases of HIV-encephalopathy when compared to that of normal brains. While very few normal astrocytes in the white matter of control biopsies showed weak cytoplasmic immunolabeling with BAG3, a robust expression of the protein was observed in the majority of reactive astrocytes in areas of gliosis in cases of HIV-encephalopathy (representative results are shown in Fig. 1, Part A). To further investigate the impact of HIV-1 expression on BAG3 expression, we investigated the level of BAG3 during the course of viral infection. To this end, the human lymphocytic cell line, SupT1, was infected with HIV-1 and BAG3 expression was monitored by Western blot. As seen in Figure 1 (Part B) a drastic increase in the level of BAG3 was detected at 10 and 15 days post-infection. The level of the control housekeeping gene, α -tubulin, remained constant. Similarly, infection of primary culture of microglial cells showed induction of BAG3 upon HIV-1 infection (data not shown).

Next, we sought to determine the effect of BAG3 induction on HIV-1 gene expression. In this regard, human primary microglial cells were transfected with a LTR reporter construct encompassing -120 to +66 -80 to +66 and in the absence and presence of BAG3. As shown in Figure 2 (Part B, left), expression of BAG3 decreased the level of LTR transcription from the -116 to +66 and had no effect on the activity of the promoter sequence spanning -88 to +66. This information led us to map the BAG3 responsive element to the region spanning between -116 and -80 that encompasses the κ B motif of the LTR. In the follow-up study to assess the impact of BAG3 on NF- κ B activation of the LTR, we transfected cells with the LTR promoter in the absence and presence of plasmids expressing the p65 subunit of NF- κ B and BAG3. As shown in Figure 2 (Part B, right), BAG3 expression decreased the level of p65-induced activation of the LTR, indicating that NF- κ B may serve as a target for BAG3-induced suppression of LTR activity. Interestingly, BAG3 exhibited a similar inhibitory effect on Tat-induced activation of the LTR and was able to inhibit the co-operativity of p65 and Tat on LTR transcription (Fig. 2C).

To further investigate the effect of BAG3 on p65 activation of the LTR, we developed and utilized siRNA for BAG3 expression, and demonstrated that BAG3-specific siRNA, but not scrambled siRNA, rescues the negative effect of BAG3 on p65-induced activation of LTR transcription (Fig. 3A). Expression levels of BAG3, p65, and the housekeeping Grb2 protein during these studies were verified by Western blot analysis (Fig. 3B, bottom).

BAG3 is a protein with 575 amino-acid residues and is composed of multiple domains including a region that interacts with proline-rich domain, multiple prolineand serine-rich domains, and a BAG domain near the C-terminus (Fig. 4A). We created a series of deletion

mutant constructs that permit expression of the various regions of BAG3 spanning its C-terminal domain. Our initial experiments as depicted in Figure 4A demonstrate that while full-length protein inhibits the activity of p65, removal of amino acids 502–575 and 418–575 fails to exhibit the inhibitory action on p65 function (Fig. 4A).

This observation provided the rationale to investigate the ability of BAG3 to physically interact with p65. Toward this end, we first constructed a plasmid that fused full-length BAG3 and the two deletion mutants, 1-502 and 1-418 to Myc tag. Results from transfection assays verified expression of these fusion proteins in the cells as determined by Western blot (Fig. 4B). The level of p65 protein upon expression of BAG3 remained fairly constant, suggesting that BAG3 had no effect on the expression of p65 in the cells. Results from immunoprecipitation of the protein complex, using anti-Myc tag antibody followed by Western blot using anti-p65 antibody showed interaction of full-length BAG3, but not the 1-502 and 1-418 deletion proteins with p65. These observations suggest that the physical interaction between the C-terminus of BAG3 and the p65 subunit of NF- κ B may be an important event in the observed suppressive effect of BAG3 on NF- κ B activation of the HIV-1 promoter. To further assess the consequence of p65:BAG3 association, we performed double labeling immunofluorescence for p65 and BAG3. BAG3 was detected in the cytoplasm with a perinuclear localization (Fig. 4C). Interestingly, p65 was also found predominantly in the cytoplasm of the cells where BAG3 was localized in the cytoplasm (Fig. 4C). Superimposition and deconvolution of the two fluorochromes demonstrated the co-localization of p65 and BAG3 in the cytoplasmic compartment.

These observations suggest that the interaction of BAG3 and p65 may affect the nuclear entry of p65 and that may prevent the interaction of p65 with the κ B motif, an essential step for p65 to exert its transcriptional activity. Thus, in the next series of experiments, we performed EMSA and ChIP assays to test p65 association with the κ B DNA sequence in the absence and presence of BAG3. To this end, protein extracts from cells transfected with plasmids expressing full-length BAG3 or BAG3 1-502, either alone or in combination with NF- κ B p65 were prepared and used in the in vitro DNA-binding assay. A [³²P]-labeled synthetic oligonucleotide DNA probe representing the κ B consensus site derived from the LTR promoter was used as probe. The DNA:protein complexes were resolved on a native polyacrylamide gel. As shown in Figure 5A, the formation of a p65-containing nucleoprotein complex, depicted with an arrow, was increased in extracts from cells transfected with p65-expressing plasmid (compare lanes 1 and 2). Co-production of p65 with full-length BAG3 in the cells decreased the level of p65 association with the κ B DNA probe (compare lanes 2 and 4), indicating that BAG3 may affect the ability of p65 to interact with its DNA motif. Nuclear extracts from cells transfected with BAG3 1-502 mutant showed a much less negative effect compared to full-length BAG3 on DNA-binding activity of p65 (compare lanes 2 and 6). To further demonstrate the in vivo association of BAG3 protein with p65, we performed ChIP assays using HeLa cells stably transfected with the HIV-1 LTR construct. In accord with the in vitro DNA-binding assays, results from PCR amplification of DNA from the immunocomplex obtained with anti-p65 antibody displayed a decrease in p65 association with the LTR DNA in cells expressing BAG3 (Fig. 5, Part B). The control IgG antibody showed no signal for p65:DNA interaction. As shown in Figure 5C, the immunocomplex pulled down with anti-p65 antibody, but not with the control antibody, contained p65, verifying the specificity of this observation. These data suggest that association of BAG3 with p65 interferes with its DNA-binding activity.

To complete its infection cycle, at the immediate stage of infection, HIV-1 relies solely on host factors to stimulate its transcription at the basal level in order to express several regulatory factors including Tat. Once Tat is produced, the virus proceeds with the subsequent stages at the early and late phase of infection through co-operativity with several host regulatory proteins. As early cell death, usually by apoptosis, is a cell defense against virus that may not

be beneficial for the successful completion of the replication cycle, the virus may utilize a variety of mechanisms to maintain cells in a functional state and prevent cells from entering into the apoptotic cycle. One of the host proteins that responds to stress such as viral infection is the anti-apoptotic BAG3 protein. Earlier studies revealed an increase in BAG3 mRNA in peripheral blood of macaques infected with a SIV/HIV-1 chimeric virus during the course of a 5-week period (Bosinger et al., 2004). Here, we demonstrate that in brain tissue of patients with HIV-1 encephalopathy, the level of BAG3 is elevated and that in vitro infection of cells by HIV-1 causes enhancement of the BAG3 expression. While the precise mechanisms underlying this event remain unknown, results from an earlier study indicate that the increase in BAG3 protein levels can be mediated by the activity of HSF1, a transcription factor that plays a pivotal role in stress response (Pirkkala et al., 2001). Evidently, HSF1 can bind to heat shock elements that are found in the BAG3 promoter and stimulate BAG3 expression (unpublished data). The question that is addressed in this report relates to the impact of BAG3 upregulation on viral gene expression. Our results identified the p65 subunit of NF- κ B as a potential target for BAG3, which through its association can suppress the activity of p65. This is an important event as expression of the HIV-1 genome relies on p65 function. It is evident that BAG3 co-localizes with p65 in the cytoplasm and blocks p65 association with its DNA motif, an important event for p65 transcriptional activity. Moreover, our results demonstrate that BAG3 can block the functional synergism between p65 and Tat protein, and thus decreases the activity of the HIV-1 promoter in microglial cells. Thus, it is evident that the induction of BAG3 upon HIV-1 infection can have an impact on viral gene expression at the immediate early (absence of Tat) and the early (presence of Tat) stages of infection. These observations may provide a new avenue for exploring the use of BAG3 as a tool for suppression of HIV-1 gene expression.

ACKNOWLEDGMENTS

The authors thank the members of the Department of Neuroscience and Center for Neurovirology for their insightful discussion and for sharing reagents, we particularly thank Dr. M. White and Dr. B. Sawaya for their useful comments. We also thank C. Schriver for editorial assistance. This work was partially made possible by grants awarded by NIH to KK and SA.

Contract grant sponsor: NIH/NINDS; Contract grant numbers: P01 NS43980, P01 NS30916.

LITERATURE CITED

- Albright AV, Soldan SS, Gonzalez-Scarano F. Pathogenesis of human immunodeficiency virus-induced neurological disease. *J NeuroVirol* 2003;9:222–227. [PubMed: 12707852]
- Amini S, Clavo A, Nadruga Y, Giordano A, Khalili K, Sawaya BE. Interplay between cdk9 and NF- κ B factors determines the level of HIV-1 gene transcription in astrocytic cells. *Oncogene* 2002;21:5797–5803. [PubMed: 12173051]
- Antoku K, Maser RS, Scully WJ Jr, Delach SM, Johnson DE. Isolation of Bcl01 binding proteins that exhibit homology with BAG-1 and suppressor of death domains proteins. *Biochem Biophys Res Comm* 2001;286:1003–1010. [PubMed: 11527400]
- Beere HM. Death versus survival: Functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J Clin Invest* 2005;115:2633–2699. [PubMed: 16200196]
- Bonelli P, Putrella A, Rosati A, Romano MF, Lerose R, Pagliuca MG, Amelio T, Festa M, Martire G, Venuta S, Turco MC, Leone A. BAG3 protein regulates stress-induced apoptosis in normal and neoplastic leukocytes. *Leukemia* 2004;18:358–360. [PubMed: 14628070]
- Bosinger SE, Hosiawa KA, Cameron MJ, Persad D, Ran L, Xu L, Boulassel MR, Parentau M, Fournier J, Rud EW, Kelvin DJ. Gene expression profiling of host response in models of acute HIV infection. *J Immunol* 2004;173:6858–6863. [PubMed: 15557180]
- Briknarova K, Takayama S, Brive L, Havert ML, Knee DA, Velasco J, Homma S, Cabezas E, Stuart J, Hoyt DW, Satterthwait AC, Llinas M, Reed JC, Ely KR. Structural analysis of BAG1 cochaperone

- and its interactions with Hsc70 heat shock protein. *Nat Struct Biol* 2001;8:349–352. [PubMed: 11276257]
- Briknarova K, Takayama S, Homma S, Baker K, Cabezas E, Hoyt DW, Li Z, Satterthwait AC, Ely KR. BAG4/SODD protein contains a short BAG domain. *J Biol Chem* 2002;277:31172–31178. [PubMed: 12058034]
- Chen L, Wu W, Dentshev T, Yong Z, Wang J, Tsui I, Tobias JW, Bennett J, Baldwin D, Dunaief JL. Light damage induced changes in mouse retinal gene expression. *Exp Eye Res* 2004;79:239–247. [PubMed: 15325571]
- Chipytisina G, Sawaya BE, Khalili K, Amini S. Cooperativity between Rad51 and C/EBP family transcription factors modulates basal and Tat-induced activation of the HIV-1 LTR in astrocytes. *J Cell Physiol* 2006;207:605–613. [PubMed: 16511829]
- Chroboczek J, Gout E, Favier AL, Galinier R. Novel partner proteins of adenovirus penton. *Curr Top Microbiol Immunol* 2003;272:37–55. [PubMed: 12747546]
- Cole, R.; Vellis, J. *Protocols for neural cell culture*. Humana Press; Totowa, NJ: 1997. p. 117-130.
- Cullen BR. The HIV-1 Tat protein: An RNA sequence-specific processivity factor. *Cell* 1990;63:655–657. [PubMed: 2225069]
- Desai-Yajnik V, Samuels H. The NF- κ B and Sp1 motifs of the human immunodeficiency virus type 1 long terminal repeat function as novel thyroid hormone response elements. *Mol Cell Biol* 1993;13:5057–5069. [PubMed: 8393143]
- Dong H, Wade M, Williams A, Lee A, Douglas GR, Yauk C. Molecular insight into the effects of hypothyroidism on the developing cerebellum. *Biochem Biophys Res Comm* 2005;330:1182–1193. [PubMed: 15823568]
- Doong H, Price J, Kim YS, Gasbarre C, Probst J, Lotta LA, Blanchette J, Rizzo K, Kohn E. CAIR-1/BAG-3 forms an EGF-regulated ternary complex with phospholipase c-gamma and hsp70/hsc70. *Oncogene* 2000;19:4385–4395. [PubMed: 10980614]
- Doong H, Vrailas A, Kohn EC. What's in the 'BAG'?—A functional domain analysis of the BAG-family proteins. *Cancer Lett* 2002;188:25–32. [PubMed: 12406544]
- Doong H, Rizzo K, Fang S, Kulpa V, Weissman AM, Kohn EC. CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation: Accumulation of poly-ubiquitinated Hsp90 client proteins. *J Biol Chem* 2003;278:28490–28500. [PubMed: 12750378]
- Everall IP. Neuronal damage—Recent issues and implications for therapy. *J Neurovirol* 2000;6(Suppl 1):S103–S105. [PubMed: 10871773]
- Gabuzda D, Wang J. Chemokine receptors and mechanisms of cell death in HIV neuropathogenesis. *J Neurovirol* 2000;6(Suppl 1):S24–S32. [PubMed: 10871762]
- Jones G, Power C. Regulation of neural cell survival by HIV-1 infection. *Neurobiol Dis* 2006;21:1–17. [PubMed: 16298136]
- Kaltschmidt B, Widera D, Kaltschmidt C. Signaling via NF- κ B in the nervous system. *Biochem Biophys Acta* 2005;1745:287–299. [PubMed: 15993497]
- Kundu M, Sharma S, De Luca A, Giordano A, Rappaport J, Khalili K, Amini S. HIV-1 Tat elongates the G1 phase and indirectly promotes HIV-1 gene expression in cells of glial origin. *J Biol Chem* 1998;273:8130–8136. [PubMed: 9525916]
- Lee J, Takahashi T, Yasuhara N, Inazawa J, Kamada S, Tsujimoto Y. Bcl-2, a bcl-2-binding protein that synergizes with bcl-2 in preventing cell death. *Oncogene* 1999;18:6183–6190. [PubMed: 10597216]
- Liao Q, Ozawa F, Friess H, Zimmermann A, Takayama S, Reed JC, Kleeff J, Buchler MW. The anti-apoptotic protein BAG-3 is overexpressed in pancreatic cancer and induced by heat stress in pancreatic cancer cell lines. *FEBS Lett* 2001;503:151–157. [PubMed: 11513873]
- Lifson JD, Reyes GR, McGrath MS, Stein BS, Engleman EG. AIDS retrovirus induced cytopathology: Giant cell formation and involvement of CD4 antigen. *Science* 1986;232:1123–1127. [PubMed: 3010463]
- Morris KV, Rossi JJ. Anti-HIV-1 gene expressing lentiviral vectors as an adjunctive therapy for HIV-1 infection. *Curr HIV Res* 2004;2:185–191. [PubMed: 15078182]
- Mufson RA, Myers C, Turpin JA, Meltzer M. Phorbol ester reduces constitutive nuclear NF κ B and inhibits HIV-1 production in mature human monocytic cells. *J Leukoc Biol* 1992;52:637–664. [PubMed: 1464736]

- Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* 1987;326:711–713. [PubMed: 3031512]
- Ouivy V, Van Lint C. Regulation at multiple levels of NF-kappaB-mediated transactivation by protein acetylation. *Biochem Pharmacol* 2004;68:1221–1229. [PubMed: 15313420]
- Pagliuca MG, Leroise R, Cigliano S, Leone A. Regulation by heavy metals and temperature of the human BAG-3 gene, a modulator of Hsp70 activity. *FEBS Lett* 2003;541:11–15. [PubMed: 12706811]
- Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ. A cooperative interaction between NFkB and Spl is required for HIV-1 enhancer activation. *EMBO J* 1993;12:3551–3558. [PubMed: 8253080]
- Petito CK. Human immunodeficiency virus type 1 compartmentalization in the central nervous system. *J Neurovirol* 2004;10(Suppl 1):21–24. [PubMed: 14982735]
- Pirkkala L, Nykanen P, Sistonen L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J* 2001;15:1118–1131. [PubMed: 11344080]
- Romano MF, Festa M, Pagliuca G, Leroise R, Bisogni R, Chiurazzi F, Storti G, Volpe S, Venuta S, Turco MC, Leone A. BAG3 protein controls B-chronic lymphocytic leukaemia cell apoptosis. *Cell Death Differ* 2003a;10:383–385. [PubMed: 12700638]
- Romano MF, Festa M, Petrella A, Rosati A, Pascale M, Bisogni R, Poggi V, Kohn EC, Venuta S, Turco MC, Leone A. BAG3 protein regulates cell survival in childhood acute lymphoblastic leukemia cells. *Cancer Biol Ther* 2003b;2:508–510. [PubMed: 14614315]
- Stout JC, Ellis RJ, Jernigan TL, Archibald SL, Abramson I, Wolfson T, et al. Progressive cerebral volume loss in human immunodeficiency virus infection: a longitudinal volumetric magnetic resonance imaging study, HIV Neurobehavioral Research Center Group. *Arc Neurol* 1998;55:161–168.
- Sweet T, Sawaya BE, Khalili K, Amini S. Interplay between NFkB and NF-kB modulates Tat activation of the LTR. *J Cell Physiol* 2005;204:375–380. [PubMed: 15887232]
- Takayama S, Reed JC. Molecular chaperone targeting and regulation by BAG family proteins. *Nat Cell Biol* 2001;3:E237–E241. [PubMed: 11584289]
- Takayama S, Xie Z, Reed JC. An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *J Biol Chem* 1999;274:781–786. [PubMed: 9873016]
- Taylor JP, Khalili K. Activation of HIV-1 transcription by Tat in cells derived from the CNS: Evidence for the participation of NF-kappa B. *Adv Neuroimmunol* 1994;4:291–303. [PubMed: 7874398]
- Torres-Munoz J, Stockton P, Tacoronte N, Roberts B, Maronpot RR, Petito CK. Detection of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains by laser capture microdissection. *J Neuropathol Exp Neurol* 2001;60:885–892. [PubMed: 11556545]
- Trillo-Pazos G, Diamanturos A, Rislove L, Menza T, Chao W, Belem P, Sadiq S, Morgello S, Sharer L, Volsky DJ. Detection of HIV-1 DNA in microglia/macrophages, astrocytes and neurons isolated from brain tissue with HIV-1 encephalitis by laser capture microdissection. *Brain Pathol* 2003;13:144–154. [PubMed: 12744468]
- Trujillo JR, Jaramillo-Rangel G, Ortega-Martinez M, Penalva de Oliveira AC, Vidal JE, Bryant J, Gallo RC. International NeuroAIDS: Prospects of HIV-1 associated neurological complications. *Cell Res* 2005;15:962–969. [PubMed: 16354575]
- Yong, VW.; Antel, JP. Culture of Glial Cells From Human Brain Biopsies. In: Federoff, S.; Richardson, A., editors. *Protocols for Neural Cell Culture*. Humana Press; Totowa, NJ: 1997. p. 157
- Young DF, Andrejeva L, Livingstone A, Goodbourn S, Lamb RA, Collins PL, Elliott RM, Randall RE. Virus replication in engineered human cells that do not respond to interferons. *J Virol* 2003;77:2174–2181. [PubMed: 12525652]

Fig. 1.

Detection of BAG3 in HIV-1-associated encephalopathy during the course of HIV-1 infection. **A:** Astrocytes in the white matter of normal brain (original magnification 400 $\times$) and in an area of gliosis of a case of HIV-encephalopathy (original magnification 200 $\times$) were analyzed by immunohistochemistry (IHC) with an anti-BAG3 polyclonal antibody. **B:** SupT1 cells in the log phase of growth were infected with JR-FL of HIV and harvested on days 5, 10, and 15. Five micrograms of total cell lysates were examined for the amount of BAG3, and α -tubulin was used as a control to monitor equal loading conditions.

Fig. 2.

Effect of BAG3 overexpression on HIV-1 LTR is dependent on κ B DNA-binding motifs. **A:** Schematic illustration of HIV-1 LTR representing two copies of the κ B domain, three GC-rich motifs, TATA box, and the TAR region, all spanning within -120 to +66. An arrow points to the transcription start site (+1), and the direction of LTR transcription. **B:** Human microglial cells were transfected with plasmids containing the sequences -120 to +66 and -80/+66 of the HIV-1 LTR promoter. CMV-p65- and CMV-BAG3-expressing plasmids were used in co-transfection and luciferase activity was determined as described in the Materials and Methods Section. **C:** Human microglial cells were co-transfected with a luciferase reporter plasmid containing the sequence from nucleotides -120 to +66 of LTR and various combinations of

CMV-p65-, CMV-Tat-, and CMV-BAG3-expressing plasmids. Luciferase activity was measured after 48 h in duplicates. Bar graphs depict the means $\pm$ standard deviations of fold increases with respect to promoter basal activity. Similar results were obtained in other two independent experiments.

Fig. 3.

Suppression of BAG3 by siRNA restores the ability of p65 to enhance LTR activity. **A:** U87MG cells were transfected with a plasmid overexpressing BAG3 and with a specific siRNA targeting BAG3 mRNA or a Scrambled siRNA, together with a CMV-p65 plasmid. Luciferase activity was determined as described in Materials and Methods section. **B:** BAG3 and NFκB p65 protein levels were assessed by Western blot and Grb2 was used as a control to monitor equal loading conditions.

Fig. 4. Effect of BAG3 deleted mutants on HIV-1 LTR activation mediated by p65. **A:** Structural organization of full-length BAG3 and the effect of BAG3 and its mutants on p65 activation of the LTR. U87MG cells were transfected as described in Experimental Procedures with a plasmid containing the sequence from nucleotides -120 to +66 of LTR. Cells were transfected with an NF- κ B p65-expressing plasmid and BAG3 deletion mutant plasmids. Luciferase activity was determined; the percentages of inhibition of p65 activity on HIV-1 LTR promoter by BAG3 FL protein and by the deletion mutants were calculated. **B:** Interaction of BAG3 and its mutants with p65. U87MG cells were transfected with the BAG3 FL plasmid and the deletion mutants. After 48 h cells were harvested and whole cell lysate were used in a co-

immunoprecipitation assay using a Myc-Tag antibody that detects full-length BAG3 (BAG3 FL) and its mutants BAG3 1-502 and BAG3 1-418. **C:** IHC with a fluorescein-tagged secondary antibody demonstrated the presence of BAG3 in the cytoplasm of astrocytic cells. The NF- κ B p65 subunit was also found in the cytoplasm of neoplastic astrocytes. Double labeling demonstrated the co-localization of both proteins in the cytoplasmic compartment of neoplastic cells (all parts: original magnification 1,000 $\times$).

Fig. 5.

Suppression of NF- κ B p65 binding to the κ B motif of the HIV-1 promoter by BAG3. **A:** Nuclear extracts from cells expressing BAG3, BAG3 (1-502) and p65 were incubated with a [32 P]-labeled probe containing the nucleotide sequence of the κ B-binding site on LTR. The nucleoprotein complexes were analyzed by native PAGE. Arrows depict the formation of the p65: κ B nucleoprotein complex. **B:** Chromatin immunoprecipitation assay was performed using a p65-specific antibody or control sera. Each sample was used in PCR using specific primers that flank the κ B motif of the LTR. The control sera and input were used as negative and positive controls. **C:** A fraction from the eluted complexes was analyzed by Western blot to verify the presence of p65 in the samples.