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# Co-Chaperone BAG3 and Adenovirus Penton Base Protein Partnership

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## ABSTRACT

The BAG family of Hsp70/Hsc70 co-chaperones is characterised by the presence of a conserved BAG domain at the carboxyl-terminus. BAG3 protein is the only member of this family containing also the N-terminally located WW domain. We describe here the identification of adenovirus (Ad) penton base protein as the first BAG3 partner recognising BAG3 WW domain. Ad penton base is the viral capsid constituent responsible for virus internalisation. It contains in the N-terminal part two conserved PPxY motifs, known ligands of WW domains. In cells producing Ad penton base protein, cytoplasmic endogenous BAG3 interacts with it and co-migrates to the nucleus. Preincubation of BAG3 with Ad base protein results in only slight modulation of BAG3 co-chaperone activity, suggesting that this interaction is not related to the classical BAG3 co-chaperone function. However, depletion of BAG3 impairs the cell entry of the virus and viral progeny production in Ad-infected cells, suggesting that the interaction between virus penton base protein and cellular co-chaperone BAG3 positively influences virus life cycle. These results thus demonstrate a novel host–pathogen interaction, which contributes to the successful infectious life cycle of adenoviruses. In addition, these data enrich our knowledge about the multifunctionality of the BAG3 co-chaperone. J. Cell. Biochem. 111: 699–708, 2010. 

**KEY WORDS:** CHAPERONES; ADENOVIRUS; PENTON BASE PROTEIN; WW DOMAIN; PPXY MOTIF; VIRAL PROGENY

Heat-shock proteins (Hsps), induced in cells in response to various types of stress, help cells resist stress-induced damage. Under normal conditions, Hsps are involved in various cell functions such as proper folding of nascent polypeptide chains, protein translocation across cell membranes, proteasome-mediated protein degradation and assembly and disassembly of multiprotein complexes. These diverse functions of Hsps are fulfilled through interaction with multiple target proteins, thereby modulating target protein activity by changing protein conformation and/or their localisation. To accomplish these functions, Hsps collaborate with co-chaperone partners, which regulate the enzymatic activity of Hsps and guide them to appropriate partner proteins.

The co-chaperones of Hsp70 protein can be divided into three families: DnaJ/Hsp40, tetratricopeptide repeat (TPR) and BAG families, each with different modular structures [for review, see Takayama et al., 2003]. In particular, the BAG family (six members in human genome) is characterised by the presence at the carboxyl-terminus of an approximately 80 amino acid BAG domain that has the ability to bind to the ATPase domain of Hsp70 and Hsc70 [Takayama et al., 1997; Antoku et al., 2001]. This interaction allows BAG family proteins to modulate the chaperone activity of Hsp70 [Lee et al., 1999; Takayama et al., 1999]. The BAG domain forms a three-helix bundle, which upon binding induces a conformational switch in the ATPase domain of Hsc70 and of bacterial Hsp70

Abbreviations used: base, Ad2 penton base protein; CBB, Coommassie Brilliant Blue; Dd, dodecahedron, subviral Ad3 particle composed of 12 penton bases; FCS, foetal calf serum; MOI, multiplicity of infection, amount of infectious virus per 1 cell; pfu, plaque forming units; PI, propidium iodide; PPxY motif, motif composed of proline–proline–amino acid–tyrosine.

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analogue DnaK; this change in conformation being incompatible with the nucleotide binding results in the ATP-dependent release of substrate from the chaperone [Gassler et al., 2001]. The amino-terminal regions of BAG proteins are not homologous, which enables them to direct Hsp70/Hsc70 proteins to various targets and cellular locations.

BAG3 (Bcl-2-associated athanogene 3), is a member of BAG family co-chaperones that besides conserved C-terminal BAG domain contains several PXXP motifs typical of SH3 domain-binding proteins [Birge et al., 1996] and one N-terminal WW domain potentially able to bind various partners. No partners of BAG3 interacting through its WW domain have been identified as yet, but BAG3 has been observed to bind through its PXXP motif the SH3 domain of phospholipase C-γ (PLC-γ), forming an epidermal growth factor (EGF)-regulated ternary complex with Hsp70/Hsc70 and PLC-g [Doong et al., 2000]. These data suggest that BAG3 may be a multifunctional signalling protein linking Hsp70/Hsc70 with other pathways necessary for activation of the epidermal growth factor receptor (EGFR) tyrosine kinase signalling, which play a key role in the regulation of cell proliferation, survival and differentiation. It has been reported recently that the molecular chaperone HspB8 forms a stable complex with BAG3 through two conserved in BAG3 Ile-Pro-Val (IPV) motifs and that this complex is involved in protein quality control [Fuchs et al., 2010].

Increases in cellular level of BAG3 protein have been noted in rat astrocytes after brain injury or upon ischaemia [Lee et al., 2002]. BAG3 induction was also observed upon heat and metal exposure in HeLa cells, with accumulation kinetics similar to that of Hsp70 [Pagliuca et al., 2003]. BAG3 (named earlier Bis) was discovered as an interacting partner of Bcl-2, and shown to suppress apoptosis [Lee et al., 1999; Antoku et al., 2001]. In this regard, BAG3 overexpression can decrease Bax- or Fas-induced apoptosis in human epithelial cells [Lee et al., 1999]. In contrast, an antisense-mediated decrease in BAG3 enhances the apoptotic response to oxidative stress and to chemotherapy in neoplastic leukocytes and reduces tumour growth in mouse models [Ammirante et al., 2010]. Expression of BAG3 widespread in mice and humans, is highest in muscle tissues [Lee et al., 1999], and is quite pronounced in developing rat brainstem and spinal cord [Choi et al., 2009]. BAG3 is an essential protein, since BAG3-deficient mice show stunted growth and fulminant myopathy and die within 4 weeks of birth [Homma et al., 2006]. It has been shown recently, that when mutated in one of IPV motifs (see above and Fig. 1B), BAG3 is implicated in dominant childhood muscular dystrophy [Selcen et al., 2009]. Some data show that BAG3 may facilitate the disposal of aggregation-prone proteins [Carra et al., 2008; Fuchs et al., 2010] and is involved in maintaining protein homeostasis during aging [Gamerdinger et al., 2009]. These observations together highlight the pro-survival activity of BAG3.

The intracellular localisation of BAG family co-chaperones is probably diverse. The best-known member of the BAG family, BAG1, was initially observed predominantly in the cytosol [Lee et al., 1999]. Later, BAG1 isoforms were discovered that preferentially localise to nuclei, in both healthy and pathological human tissues [Takayama et al., 1998; Yamauchi et al., 2001; Liman et al., 2005]. In contrast, BAG1ΔC, a truncated protein devoid of C-terminally located BAG domain and thus unable to interact with Hsp70, was expressed exclusively in the cytosol [Liman et al., 2005]; interaction with Hsp70 permit BAG1 transfer to the nucleus. Similar data concerning BAG3 are rather scarce. BAG3 has been shown to have an exclusively cytoplasmic localisation in unstressed cells and a rather pronounced concentration in the rough endoplasmic reticulum upon treatment with heavy metals [Pagliuca et al., 2003]. In muscle, BAG3 localises to sarcomeres [Homma et al., 2006].

Adenovirus (Ad) penton base protein is the constituent of the virus capsid that is responsible for virus internalisation [Wickham et al., 1993] and endosomal release [Seth, 1994]. During expression library screening for penton base partners one of the proteins identified was the BAG3. Here we demonstrate the interaction between BAG3 and the Ad penton base and describe the localisation of both proteins in human cells. Our data imply that interaction with BAG3 positively modulates Ad life cycle, and is in particular implicated in virus cell entry. These data enrich our knowledge about the multifunctionality of the BAG3 co-chaperone.

## MATERIALS AND METHODS

### CELLS, CLONES, ANTIBODIES, VIRUS

HeLa cells were cultured at 37°C, under 5% $CO_2$ atmosphere, in EMEM (BioWhittaker) supplemented with 10% foetal calf serum. BAG3 and its C-terminal deletion mutant (BAG3ΔC) were generated as described by Takayama et al. [1999]. Appropriate cDNA fragments were subcloned into the pGEX4T-1 (GE Healthcare), pcDNA3-myc (Invitrogen) and pEGFPC2 (Clontech) vectors using *Eco*RI and *Xho*I restriction sites. Full-length Ad2 penton base and penton base mutants ΔPY1 and PY2mut were cloned into pcDNA3.1 (Invitrogen) as described by Galinier et al. [2002].

For Ad2 and Ad3 penton base recognition anti-Ad3 dodecahedron polyclonal serum was used at dilutions 1:40,000 for Western blot and 1:800 for confocal microscopy. For penton base immunoprecipitation, the anti-Ad2 base protein polyclonal serum, kind gift of Prof. P. Boulanger, was used. Monoclonal antibodies anti-c-myc HRP-conjugated (Roche) and monoclonal anti-GST (Sigma) were used for BAG3 recognition on Western blots, both at 1:1,000. For immunoprecipitation anti-c-myc monoclonal antibody (Roche) or 9E10 MAb from Abcam were used. Western blots were developed with anti-BAG3 at 1:250 (Takayama clone 2, recognising both transfected and endogenous BAG3 [Takayama et al., 1999]). Anti-calnexin (from Stressgen, a kind gift of Corinne Albiges-Rizo) was used at 1:1,000. For Hsp70 detection and immunoprecipitation the polyclonal anti-Hsp72 antibody SPA-812 from Stressgen was used (at 1:5,000 for Western blot). HRP-conjugates of secondary antibodies (Jackson) were used for Western blot at 1:5,000. Immunoprecipitations were carried out with 1 μl antibody per 20 μl of protein A/G-agarose suspension (Amersham Pharmacia).

Ad2 wild-type and Ad5luc were propagated in HeLa and HEK293 cells, respectively, infected at MOI 1 for 72 h. Virus stocks were purified from infected cells lysates, by double banding on CsCl gradient according to Kanegae et al. [1994]. The virus band was dialysed against 20 mM Tris, pH 7,4, containing 150 mM NaCl and stored with 20% glycerol at −20°C.

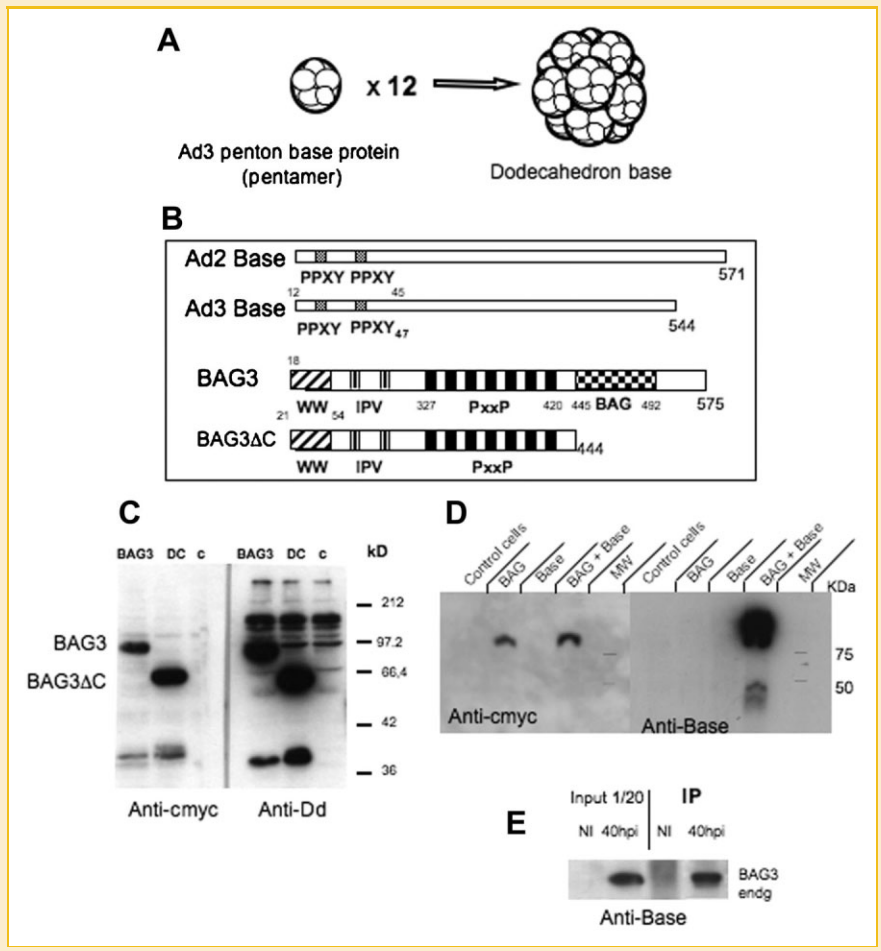


Fig. 1. Interaction of Ad2 penton base protein with BAG3. A: Diagram of Ad3 penton base and dodecahedron base. B: Diagram of Ad2 and Ad3 penton base proteins as well as human BAG3 and C-terminally truncated BAG3ΔC. Functional domains of BAG3 are marked. C: Interaction of Ad3 penton base with BAG3 in vitro revealed by overlay. HeLa cells were transfected with c-myc-tagged BAG3 or BAG3ΔC, as described in the Materials and Methods Section. Cell lysates were resolved on SDS/PAGE and the BAG3 proteins were revealed by Western blot with anti-c-myc serum (left part). A similar membrane was overlayed with Ad3 dodecahedra and analysed with anti-Dd antibody (right part), as described in the Materials and Methods Section. D: Interaction of Ad3 penton base with BAG3 in vitro revealed by Immunoprecipitation in vitro. E: Interaction of Ad3 penton base with BAG3 in vivo. HeLa cells were infected with wt Ad2. Cell lysates obtained at 40 hpi were immunoprecipitated with anti-BAG3 recognising endogenous protein and visualised on Western blot with anti-base antibody. NI, non-infected cells.

### PROTEINS

Ad3 dodecahedra (Dd) built of 12 penton bases were produced using a baculovirus system and purified on a sucrose density gradient as described by Fender et al. [1997]. The preparations of Dd used for the assays described in Figure 2 were further purified on Q-Sepharose column. GST-BAG3, GST-BAG3ΔC and GST expressed in DH5α grown at 16°C and induced with 0.5 mM IPTG overnight, were purified from a sonicated bacteria pellet on a glutathione–sepharose column (Amersham Biosciences), using for elution 10 mM glutathione in 50 mM Tris, pH 7.5, containing 200 mM NaCl.

### SCREENING OF THE EXPRESSION LIBRARY

A human lung cDNA expression library in lambda gt11 (Clontech) was used throughout this work. The screening for protein–protein interactions was performed with adenovirus dodecahedra, developed with the polyclonal antibody against Ad3 penton diluted at 1:4,000 as described in Galinier et al. [2002]. Positive clones were plaque-purified; DNA was isolated from phage lysates using a Qiagen lambda midi kit and sequenced.

### TRANSFECTION, AD INFECTION AND IMMUNOPRECIPITATION

HeLa cells ($5 \times 10^5$ cells in 35 mm dish) were transfected using 3 μl Fugene for 1 μg of DNA, following manufacturer's protocol (Roche). When necessary, HeLa cells transfected for 24 h were infected with adenovirus at MOI 5 without serum for 1 h at 37°C. After 48 h for transfection only or further 24 h growth for infection, cells were collected and lysed in 400 μl of 20 mM Tris, pH 7.5, containing 150 mM NaCl, 10 mM $MgCl_2$, 10 mM NaF, 1 mM $Na_3VO_4$, 10% glycerol, 0.1% NP40 and protease inhibitors cocktail (Roche), with one cycle of freeze-thawing. Supernatant was recovered by centrifugation and precleared with 5% (v/v) of Protein A/G-Sepharose (Amersham Biosciences) for 2 h at 4°C. Immunoprecipitation was performed for 4 h at 4°C at constant rotation with 5% (v/v) Protein A/G-Sepharose (Amersham Pharmacia) containing

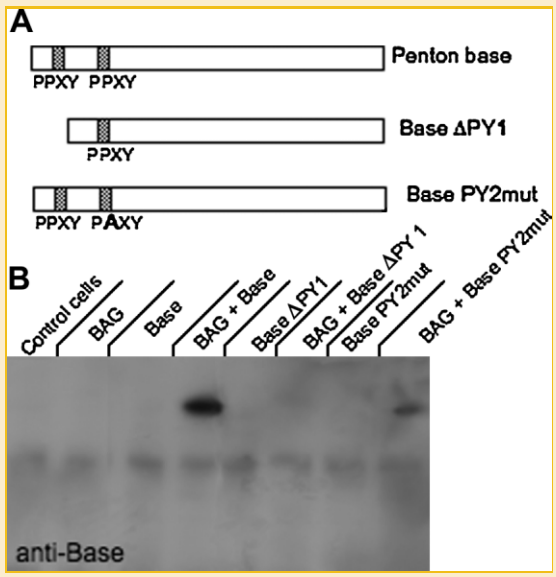


Fig. 2. The first N-terminal PPxY motif of base protein is involved in the interaction with BAG3. HeLa cells were transfected with plasmids coding for the indicated proteins. Lysates were immunoprecipitated with anti-c-myc bringing down BAG3 and developed with anti-Dd antibody as described in the Materials and Methods Section.

anti-c-myc, anti-BAG3, anti-Hsp70 or anti-penton base (1 μl antibody/20 μl beads, prefixed for 2 h at 4°C).

### INTERACTION IN VITRO AND WESTERN BLOTTING

HeLa cells were transfected as above for 48 h. Collected cells were lysed in 100 μl of lyse buffer of luciferase kit (Promega). One-fifth part was analysed by SDS/PAGE and electrotransferred to the PVDF Immobilon membrane (Millipore). The membrane, saturated with Tris buffer saline (TBS) containing 0.5% Tween-20 and 5% of defatted milk (TBST/milk), was either revealed with anti-mycHRP antibody at 1:1,000 or overlaid with dodecahedron solution (1 μg in 3 ml TBST/milk) and after several washes with TBST developed, using the anti-Dd antibody at 1:40,000.

### CONFOCAL MICROSCOPY

HeLa cells were grown overnight on glass coverslips (about $10^5$ cell/$cm^2$) at 37°C under 5% $CO_2$ atmosphere in EMEM medium supplemented with 10% FCS. Coverslips were placed in 24-multiwell plates and cells were transfected with various plasmids using Fugene (3 μl Fugene/1 μg DNA per well). In cases when cells were infected with virus, Ad2 was applied at MOI 5 in 250 μl/well of EMEM without serum. After 1 h at 37°C the inoculum was removed and 750 μl EMEM containing 10% FCS was added. After indicated period of transfection or infection the medium was removed, cells on glass coverslips were rinsed with PBS, fixed in 2% paraformaldehyde in PBS for 20 min, rinsed three times with PBS and permeabilised with 0.2% Triton X-100 in PBS for 3 min. Cells were again rinsed three times with PBS, incubated with appropriate antibody in 50 μl PBS-0.05% Tween-20 and 1% BSA, and a subsequent incubation with Texas-Red secondary antibody (Jackson) diluted 1:800 in the same buffer, was performed. When needed, cell nuclei were counter-stained for 3 min with propidium iodide (5 μg/ml). Samples were observed using a microscop Optiphot II (Nikon) coupled to a laser scanning confocal apparatus MRC600 (Bio-Rad). FITC and Texas Red fluorescence was excited at 488 and 543 nm, and emission was measured at 500–530 and 565–640 nm, respectively. Image acquisition was done with software Comos, and figures were processed with Photoshop 6.0.

### EFFECT OF BAG3 DEPLETION ON AD5 INFECTION

**Virus entry—Luciferase reporter assay.** HeLa cells were infected with either BAG3 siRNA retrovirus or control retrovirus, followed by puromycin selection (1 μg/ml) to obtain permanent knockdown cells as described by Homma et al. [2006]. Purified Ad5Luc (0.5 μl) was added to BAG3-depleted or control cells ($5 \times 10^4$) plated in wells of 24-well plate. The amount of Ad5luc added corresponded to half-saturation of HeLa cells with Ad5Luc. After 24 h transfection cells ($n = 5$) were lysed with luciferase reporter buffer (Promega). Twenty microlitres of lysate were mixed with 100 μl of luciferin solution (Promega) and the luciferase activity was measured with luminometer (Biotek Synergy HT). Actin recognised with specific antibody at 1:5,000 (Calbiochem) was used as a loading control.

**Virus production.** Purified wtAd5 (0.5 μl, $5 \times 10^6$ pfu) was added to BAG3-depleted or control cells ($5 \times 10^4$) plated in 24-well plate, which amounts to MOI of 100. After 24 and 48 h, cells were lysed with RIPA buffer (100 μl), sonicated and 20 μg lysate/lane was analysed by Western blot with anti-base at (1:10,000), and anti-actin antibodies followed by anti-BAG3 (1:2,000) (in this sequence). Control cells were obtained by 24 h transfection of HEK293 with pcDNA3/Ad2 penton base expression vector [Galinier et al., 2002].

## RESULTS

### BAG3 IS A CELLULAR PARTNER OF THE ADENOVIRUS PENTON BASE

To search for cellular proteins that interact with the penton base we used Ad serotype 3 subviral particles composed of 12 penton bases, that is adenovirus dodecahedra [Fender et al., 1997, Fig. 1A] to screen a human lung expression library for interacting proteins. The screen yielded several different penton base partners, among them BAG3 protein. The common structural feature of identified penton base partners is the WW domain, one of several domains known to promote physical interaction between proteins [Sudol, 1996]. Indeed, BAG3 is the only member of the BAG family that contains a WW domain, which is located in the N-terminal part of the protein (Fig. 1B). WW domains of 25–40 amino acids contain a high proportion of hydrophobic aromatic and basic residues, two highly conserved tryptophanes at both ends and one invariable proline. These domains bind to short poly-proline motifs that differ from other poly-prolines such as ligands of the SH3 domains. Several classes of such poly-proline ligands of WW domains are known [for review, see Macias et al., 2002] and one of them, PPxY (proline-proline-amino acid–tyrosine) appears twice in the N-terminus of all sequenced penton bases.

## PENTON BASE PROTEIN INTERACTS WITH BAG3

The membrane overlay technique was initially used to confirm the interaction uncovered by the library screen. The lysate of BAG3-transfected HeLa cells contained two major bands revealed with anti-tag antibody (Fig. 1C, left panel). The larger protein recognised by antibody against BAG3 tag migrated with an apparent molecular weight of ~80 kDa rather than 64 kDa expected for BAG3; anomalous BAG3 mobility have already been noted by Lee et al. [1999]. The C-terminal BAG3 deletion mutant (BAG3ΔC, apparent MW of 50 kDa), synthesised in larger amount than full-length protein, ran at the level of 60 kDa. Both BAG3 proteins were able to attach subviral Ad3 dodecahedral penton bases particles recognised by anti-Dd antibody (Fig. 1C, right panel). The band observed above the 36 kDa MW marker in Figure 1C, is probably a proteolysis product containing the BAG3 N-terminal WW domain since it is able to interact with base protein (Fig. 1C, right panel). The bands visible above BAG3 are recognised by anti-Dd antibody in the non-transfected cells (Fig. 1C, lane c) and they might represent other cellular proteins interacting with the penton base. The thin band running at the level of complete BAG3 in lanes DC and of control possibly represents the endogenous BAG3 protein. The control blot treated with anti-Dd antibody alone did not show any bands (not shown). BAG3- or penton base-transfected cells were then immunoprecipitated with anti-c-myc tag antibody recognising overexpressed BAG3. The first membrane, developed with anti-BAG3-HRP antibody, showed correct immunoprecipitation of BAG3 (Fig. 1D, lanes BAG and BAG + Base). Revelation of the second membrane with anti-base antibody demonstrated that the Ad2 base protein was immunoprecipitated together with BAG3 protein (Fig. 1D, lane BAG + Base). In the absence of BAG3, penton base protein alone was not immunoprecipitated. Finally, to determine if this interaction exists under more physiological conditions, lysates obtained from cells infected with Ad2 for 40 h were immunoprecipitated with the antibody recognising endogenous BAG3. The antibody brought down penton base protein (Fig. 1E), which shows that penton base expressed during Ad2 infection is indeed in the complex with the endogenous BAG3. This last experiment confirms the BAG3–penton base interaction during infectious viral cycle.

Adenovirus base proteins contain two PPxY motifs (Fig. 1) and we have shown previously that the first N-terminal PPxY motif is indispensable for interaction with other penton base partners [Galinier et al., 2002]. To explore whether a similar mechanism applies to the interaction with BAG3, the experiment was performed using two mutants of the Ad2 base protein, namely ΔPY1 and PY2mut. The former had the first PPxY motif deleted while the latter retained both motifs but had a second proline of the second PPXY changed to alanine (see diagram Fig. 2A). The rationale for this last mutation is based on studies identifying this residue in the PPxY motif as indispensable for interaction with WW domains [Chen et al., 1997; Pirozzi et al., 1997]. In cells co-transfected with penton base or the base mutant genes and with the BAG3 expression plasmid, the BAG3 immunoprecipitated the native form of the penton base protein and the PY2mut, but not the base devoid of the first PPXY motif (Fig. 2B, compare lanes BAG + Base and BAG + PY2mut with the lane BAG + ΔPY1). Of note, the synthesis of N-terminally deleted base protein is always somewhat impaired (results not shown). This result suggests that also for BAG3 the first PPxY motif in the penton base is involved in the interaction.

## PENTON BASE PROTEIN PREVENTS INTERACTION OF BAG3 WITH HSP70

It is known that the C-terminal part of BAG3 interacts with Hsp70 [Takayama et al., 1997] while we show here that this is BAG3 N-terminal part that is involved in the interaction with the Ad base protein, since C-terminally deleted BAG3 still interacts with the base (Fig. 1C). It was thus of interest to determine if the ternary complex can be formed by BAG3 bearing both partners simultaneously. For this, HeLa cells were transfected with both c-myc-tagged BAG3 and Ad2 penton base-coding plasmids. Cell lysates were immunoprecipitated with anti-c-myc, anti-Hsp70 or anti-base antibodies. Interestingly, both anti-c-myc and anti-Hsp70 antibodies brought down both tagged and endogenous BAG3 (arrowheads in Fig. 3A). Both base protein and endogenous Hsp70 were pulled down together with BAG3. However, we excluded existence of a ternary complex by performing reciprocal immunoprecipitations in which the anti-Hsp70 antibody brought down both transfected and endogenous BAG3 co-chaperone, but was unable to precipitate the putative third component of the complex—penton base protein (Fig. 3A, column 'Base'). This experiment shows that BAG3 protein is involved in formation of two mutually exclusive complexes, either with Hsp70 or with Ad penton base.

BAG family proteins bind with high affinity to the ATPase domain of Hsp70 and can affect Hsp70 chaperone activity that is

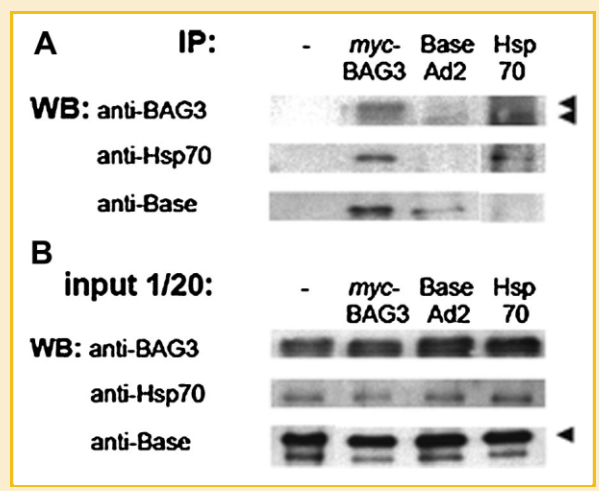


Fig. 3. BAG3 is not capable of forming ternary complex with penton base and Hsp70. HeLa cells were transfected with plasmids coding for c-myc tagged-BAG3 and Ad2 penton base. Lysates were immunoprecipitated with antibodies recognising c-myc (BAG3), Ad2 base or Hsp70, respectively. Western blots were developed with anti-BAG3 recognising both tagged and endogenous forms of the protein, anti-base and anti-Hsp70 as described in the Materials and Methods Section. Upper panel: Co-immunoprecipitation of BAG3 with either Ad2 base or endogenous Hsp70. Ad2 base forms a complex with BAG3 but not with Hsp70. Endogenous Hsp70 brings down BAG3 protein but not Ad2 base. The head arrows indicate c-myc-tagged and endogenous BAG3 proteins. Bottom panel shows proteins contained in 1/20 of lysates taken for the immunoprecipitation experiment shown in the upper panel.

controlling protein folding, To investigate whether the interaction with the penton base protein has an effect on BAG3 activity, the ATPase activity of Hsp70 as well as refolding of denatured luciferase were measured. Both BAG3 and BAG3ΔC were expressed as GST-fusion proteins and the recombinant Ad2 base protein as well as the dodecahedra (complex of 12 Ad3 bases) were obtained by expression in the baculovirus system. We observed that the interaction of both kinds of penton bases with BAG3 might somewhat modulate the activity of BAG3 as an Hsp70 co-chaperone, possibly through removal of BAG3 from the available co-chaperone pool (Suppl. Mat. Fig. 1). This result is rather obvious if we remember that BAG3 is Hsp70 co-chaperone and BAG3 in complex with penton base is unable to interact with Hsp70, as shown above (Fig. 3A).

### INTERACTION OF BAG3 PROTEIN WITH PENTON BASE IN LIVING CELL

We used the green fusion protein GFP-BAG3 and the DNA-binding fluorochrome, propidium iodide (PI) to examine the location of the BAG3 protein in adenovirus-infected cells. GFP control protein was observed uniformly throughout the cell, with somewhat pronounced concentration in the nucleus (Fig. 4A, left panel). The localisation of GFP did not change upon Ad2 infection (Fig. 4A, right panel). In contrast, GFP-BAG3 was observed predominantly in the cytoplasm, with marked concentration around the nucleus (Fig. 4B, upper left panel) and with some overlap with calnexin, an endoplasmic reticulum (ER) marker (Fig. 4B, lower panel). ER localisation of BAG3 has been observed previously upon treatment with heavy

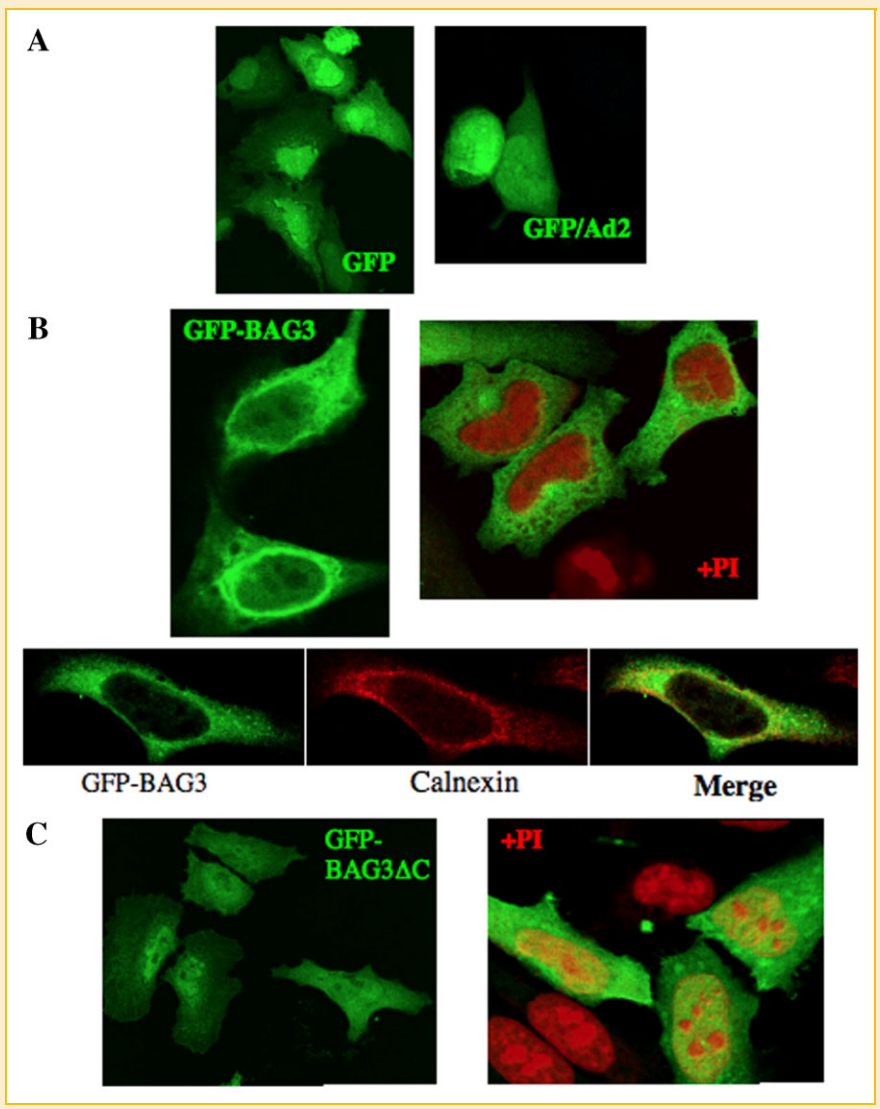


Fig. 4. Intracellular localisation of GFP, BAG3 and BAG3DC, monitored by immunofluorescence. A: HeLa cells were transfected with pGFP (left panel, in green) or transfected with pGFP and infected with Ad2 (right panel). B: Upper panel: HeLa cells were transfected with pGFP-BAG3 (in green) and cell nuclei were counterstained in red with propidium iodide (PI) in order to better show cytoplasmic localisation of BAG3. Lower panel: GFP-BAG3 is shown in green. Calnexin was revealed with anti-calnexin antibody and Texas Red-conjugated secondary antibody. C: HeLa cells were transfected with pGFP-BAG3ΔC (in green) and cell nuclei were counterstained in red with propidium iodide (PI). Confocal microscopy was performed as described in the Materials and Methods Section.

metals [Pagliuca et al., 2003]. C-terminally truncated BAG3, GFP-BAG3ΔC, was localised in both the cytoplasm and nuclei of the transfected cells but was clearly excluded from the nucleoli (Fig. 4C). This suggests that the removal of the C-terminal portion of BAG3 along with the BAG domain (amino acids 445–575) either revealed a hidden NLS (nuclear localisation signal) or, alternatively, removed a dominant cytoplasmic retention signal.

During Ad infection the penton base protein synthesised in the cytoplasm in the late part of the viral life cycle is rapidly, 1–3 min after synthesis, transported to the nucleus where the virus assembly takes place [Velicer and Ginsberg, 1970]. The mechanism of the penton base transfer to the nucleus is not clear since its sequence does not display an obvious NLS. However, the nuclear transfer of the penton base was confirmed in our hands upon the expression of the base protein alone in HeLa cells, under conditions of both transfection and virus infection (Fig. 5A). When BAG3 was expressed in the presence of the penton base, a significant portion of GFP-BAG3 was observed in the nucleus (Fig. 5B, see panels Merge). Since this protein is predominantly displayed in the cytoplasm in the absence of Ad infection (see Fig. 4B), these

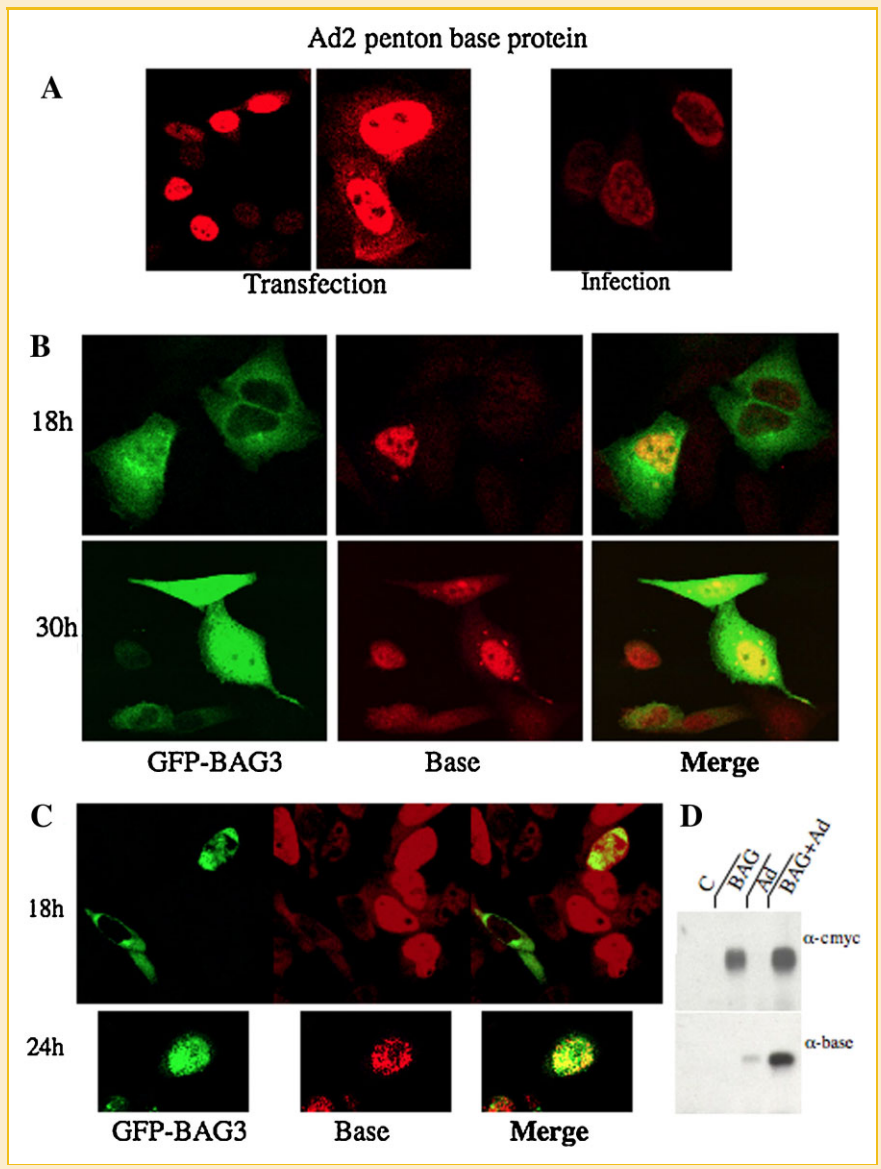


Fig. 5. Nuclear localisation of BAG3 upon expression of Ad2 penton base. The base protein is revealed with anti-Dd antibody recognising penton base and with the Texas Red-conjugated secondary antibody, while GFP-BAG3 is shown in green. A: Localisation of base protein in HeLa cells either transfected with Ad2 penton base-expression plasmid or infected with Ad2. B: Localisation of BAG3 in cells transfected with penton base. Note that cells transfected solely with BAG3 display the protein mainly in cytoplasm whereas cells expressing base protein show co-localisation in the nucleus. C: Localisation of BAG3 in cells infected with Ad2. The green stain of BAG3 clearly overlaps with the red stain of base, showing again the co-localisation of both proteins in the nucleus. D: Interaction of BAG3 with penton base in Ad2-infected cells. Upper panel: HeLa cells were transfected with c-myc-BAG3 and infected with Ad2 (BAG + Ad2). Immunoprecipitation was done with anti-c-myc. Revelation performed with anti-c-mycHRP (WB) shows comparable amounts of BAG3 in non-infected and Ad-infected cells. Column Ad (virus) shows the mobility of marker penton base protein.

observations suggest that the penton base protein, when expressed together with BAG3, is able to transfer the attached BAG3 to the nucleus during its own nuclear transport. Similar behaviour of BAG3 and base proteins was observed upon virus infection, with a remarkably high concentration of BAG3 in the nucleus of Ad2-infected cells (Fig. 5C). Fig. 5D shows the simultaneous expression of BAG3 and penton base protein in the infected cells. Taken together these experiments show that by virtue of an interaction with a viral partner capable of efficient nuclear translocation, BAG3 co-chaperone is transported into the nuclei of penton base-expressing cells, thus acquiring a new localisation upon Ad infection.

### EFFECT OF BAG3 ON ADENOVIRUS ENTRY AND MULTIPLICATION

Finally, we explored the role BAG3 plays in adenovirus life cycle. HeLa cells depleted of BAG3 using siRNA technique (Fig. 6A, left panel) were infected with the adenovirus carrying the luciferase gene, Adluc. This recombinant virus is unable to replicate in HeLa cells but translocates the virus genome to the cell nucleus and allows luciferase expression, which permits the quantification of virus entry. Depletion of BAG3 appears to be deleterious to virus entry (Fig. 6A, right panel). Next, BAG3-deleted cells were infected with Ad5 and analysed for the amount of progeny virus produced, taking the penton base production as a measure. During Ad replication penton base is one of three structural viral proteins produced in large amounts (the other two being the hexon and the fibre). BAG3-deleted cells showed reduced virus replication, which was demonstrated by the significantly lower amount of penton base protein synthesised, as observed within the first 24 h of infection (Fig. 6B). This effect is much less pronounced at 48 h, which might result from the high multiplicity of infection used in this experiment. Together, these experiments suggest that interaction of the penton base with BAG3 is implicated in the first stages of Ad infection—virus entry and nuclear translocation.

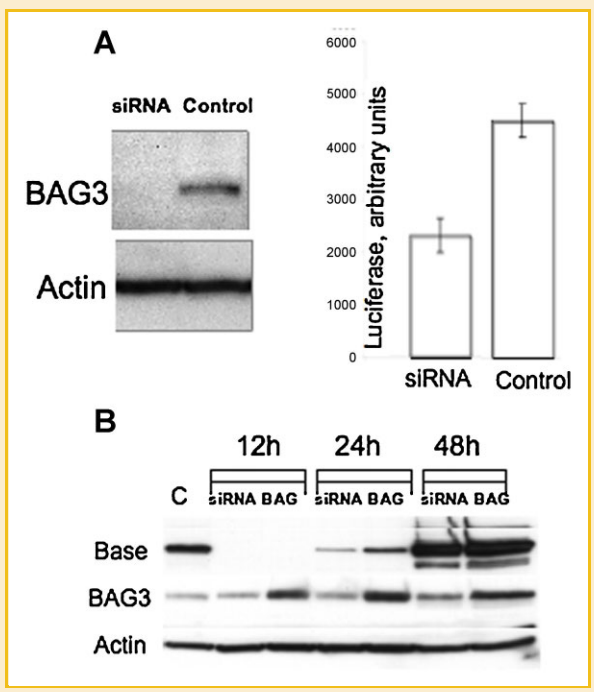


Fig. 6. Effect of BAG3 on Ad5 infection. A: BAG3 depletion results in impaired adenovirus cell entry. HeLa cells were infected with either BAG3 siRNA retrovirus or control retrovirus, followed by puromycin selection (1 μg/ml), to obtain permanent knockdown cells, as described in the Materials and Methods Section. Left panel shows BAG3 expression in siRNA-treated cells as compared with untreated cells, visualised by Western blot with anti-BAG3 antibody. Actin is shown as loading control. Right panel: BAG3-depleted or control cells were treated with Ad5Luc and 24 h later luciferase expression was measured (see the Materials and Methods Section). Luciferase activity is shown with error bar as mean ± SD (n = 5). B: BAG3 depletion results in impaired virus production measured by penton base synthesis. HeLa cells with or without BAG3 siRNA expression were infected with wtAd5 as described in the Materials and Methods Section. At indicated times, cell lysates were analysed by Western blot with anti-base, anti-BAG3 and anti-actin antibodies. Control (marked with C) contains an aliquot of HEK293 cells transfected with pcDNA3/Ad2 penton base expression vector. Actin is shown as a loading control.

## DISCUSSION

Here we show that adenovirus penton base protein interacts with BAG3 protein, both in vitro and in living cells. Ad penton base protein contains conserved N-terminal PPxY motifs and such polyproline motifs are known to bind WW domains [Chen et al., 1997]. The BAG3 is the only member of the BAG family co-chaperones containing WW domain and since the penton base devoid of first PPxY motif is unable to interact with BAG3, it can be inferred that the first PPxY domain of the penton base and the WW domain of BAG3 jointly mediate the penton base–BAG3 interaction. This is the first report on the partner interacting with BAG3 through its WW domain.

It has been shown that BAG family proteins bind with high affinity to the ATPase domain of Hsp70 and modulate its chaperone activity [Nollen et al., 2000]. In our hands, low concentrations of BAG3 stimulated both the chaperone and ATPase activity of Hsp70 while at higher concentrations it was without effect or inhibitory (see Suppl. Mat. Fig. 1). We wished to know whether the interaction with the penton base influences BAG3 function. To better understand the effect of the base protein on BAG3 co-chaperone activity, proteins derived from two different adenovirus serotypes were used—Ad2 penton base and Ad3 dodecahedron, a virus-like particle consisting of 12 bases [Fender et al., 1997]. However, both kinds of penton base proteins exerted rather weak inhibitory effects on the co-chaperone activity of BAG3 (Suppl. Mat. Fig. 1B,D). Moreover, this negative modulation could be observed only under conditions favouring the interaction between BAG3 and base protein, for example after 1 h preincubation of the two partners prior to the assay.

Interestingly, despite the fact that the interaction with base protein involves BAG3 N-terminal part, while the interaction with Hsp70 relies on the C-terminal part of BAG3, BAG3 is unable to form a ternary complex in which it attaches simultaneously to penton base and Hsp70. When no tertiary complex of Base-BAG3-Hsp70 is formed, penton base protein cannot affect Hsp70 chaperone activity by BAG3 intermediate, which explains a negligible effect of base presence on co-chaperone activity of BAG3. It seems therefore, that

in the presence of penton base and also, conceivably, upon Ad infection, the co-chaperone activity of BAG3 is detoured from the Hsp70 system toward viral life cycle. Of note, BAG3 has been recently shown to act in a non-canonical manner unrelated to the classical chaperone model, without requirement for Hsp70 and targeting fully folded substrates [Carra et al., 2008].

Ad penton base protein is a large pentameric protein, which enables virus internalisation by interacting with host integrins [Wickham et al., 1993]. This protein is also implicated in the escape of the virus from endosomes [FitzGerald et al., 1983]. In addition, since viral proteins are synthesised in the cytoplasm they have to travel to the nucleus where the assembly of Ad progeny virions takes place. It can thus be inferred that during its life cycle the penton base is involved in the host cell in a plethora of interactions that permit virus cell entry and release from endosomes, as well as viral protein synthesis, folding, oligomerisation, nuclear transport and finally virion assembly. We thought that localisation studies could be of help in elucidation of BAG3 involvement in virus life cycle. The well-studied member of a BAG co-chaperone family, BAG1, localises to both, the cytosol and nucleus [Nollen et al., 2000; Frebel et al., 2007]. However, for BAG3 we observed predominantly cytoplasmic localisation, with some overlap with an endoplasmic reticulum marker, calnexin (Fig. 4B, lower panel). In contrast, C-terminally truncated BAG3 was found in both cytoplasm and nuclei of the transfected cells (Fig. 4C). This suggests that the removal of the C-terminal portion of BAG3 (amino acids 445–575 which includes BAG domain) either revealed a hidden NLS or, alternatively, removed a dominant cytoplasmic retention signal. Indeed, the deleted C-terminal part of the BAG3 includes a strongly acidic/hydrophobic fragment 455–475 containing some conserved cytoplasmic retention regions [Bertos et al., 2004], as well as the sequence close to the consensus sequence for the nuclear export signal (NES) [Wang et al., 2004].

Remarkably, the complete BAG3 was translocated to the nucleus upon interaction with Ad base protein. Ad base protein, despite the apparent lack of NLS, translocates efficiently to the nucleus and the BAG3 protein is apparently able to piggyback with it (Fig. 5). It has been shown that interaction with Hsp70 is often sufficient for nuclear translocation of the chaperone client protein [Cripe et al., 1995; Florin et al., 2002]. As BAG3 is unable to form a ternary complex containing both penton base and Hsp70 (Fig. 3A), it appears that the interaction with Ad penton base is sufficient to result in the nuclear transfer of BAG3. To this end the virus may subvert BAG3 to ensure increase of penton base nuclear translocation. Additional experiments are needed to elucidate the nuclear role of BAG3 during viral infection, in particular since Ad assembly occurs in the nucleus.

To determine the effect of BAG3 on virus infection and at what step the partnership with BAG3 intervenes, we estimated the amount of penton base produced in cells transfected with the non-replicative Adluc (measurements of viral entry) as well as in cells infected with Ad2 upon BAG3 depletion by siRNA. Under conditions of BAG3 depletion, virus entry was diminished by about half (Fig. 6A), implying that this step is facilitated by the interaction between the penton base and BAG3. Similarly, upon decrease in BAG3 the expression of penton base protein during virus infection was diminished showing impaired virus production (Fig. 6B). It appears that Bag3 interaction with Ad penton base is implicated in the early steps of Ad infection, that is virus entry and nuclear translocation.

BAG3 has been reported to interact with enveloped viruses. Rosati et al. [2007] showed that BAG3 modestly suppresses transcription from the long terminal repeat (LTR) of HIV-1, through inhibition of interaction of p65 subunit of NF-κB with the LTR. The C-terminal fragment of 167 amino acid residues of BAG3 was mapped here as potential p65-binding domain. The observed suppression level was two- to fourfold only. In contrast, Varicella-Zoster virus (VZV) replication is strongly facilitated by the BAG3 [Kyratsous and Silverstein, 2008]; ORF29p, a latency-associated VZV protein, interacts with BAG3 and BAG3 depletion with siRNA inhibits virus replication. BAG3 devoid of WW domain was still able to bind viral protein. Similarly to our observations made for the adenovirus, BAG3 was located in cytoplasm of non-infected cells and was re-distributed to the nucleus upon VZV infection. In case of VZV, reduction of BAG3 level resulted in marked decrease in virus titre. In contrast, the spread of another herpesvirus, HSV, was completely unaffected by BAG3 depletion (op. cit.), even that recent data indicate on the positive effect of BAG3 on HSV replication at low multiplicity of infection [Kyratsous and Silverstein, 2008]. Interestingly, still another herpesvirus, EBV, via its EBNA3A oncoprotein, was able to induce mRNA of several chaperones, among them that of BAG3 [Young et al., 2008]. Further, BAG3 suppression with RNAi resulted in inhibition of a respiratory virus SARS-CoV replication [Zhang et al., 2010]. Thus BAG3 appears to be involved in several facets of host–virus interactions.

Our results on the interaction of BAG3 co-chaperone with adenovirus penton base are the first data concerning non-enveloped virus and the first identification of the BAG3 WW domain partner. These data enrich our knowledge about the multifunctionality of the BAG3 co-chaperone and in particular provide further support of a role for BAG3 as an important host modulator of virus infection.

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