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Chaperone BAG6 is dispensable for MHC class I antigen processing and presentation

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ABSTRACT

Antigen processing for direct presentation on MHC class I molecules is a multistep process requiring the concerted activity of several cellular complexes. The essential steps at the beginning of this pathway, namely protein synthesis at the ribosome and degradation via the proteasome, have been known for years. Nevertheless, there is a considerable lack of factors identified to function between protein synthesis and degradation during antigen processing. Here, we analyzed the impact of the chaperone BAG6 on MHC class I cell surface expression and presentation of virus-derived peptides. Although an essential role of BAG6 in antigen processing has been proposed previously, we found BAG6 to be dispensable in this pathway. Still, interaction of BAG6 and the model antigen tyrosinase was enhanced during proteasome inhibition pointing towards a role of BAG6 in antigen degradation. Redundant chaperone pathways potentially mask the contribution of BAG6 to antigen processing and presentation.

1. Introduction

Antigen processing and presentation in the MHC class I restricted pathway is a process comprising several subsequent steps of polypeptide processing and transportation. Generally, proteins are degraded to peptides by the proteasome, loaded onto MHC class I molecules, and presented as a complex to cytotoxic T lymphocytes (CTLs) at the cell surface (Rock and Goldberg, 1999). Based on the discovery of an intimate connection between antigen presentation and protein synthesis Yewdell and colleagues postulated the defective ribosomal product (DRiP) hypothesis (Yewdell et al., 1996). DRiPs are thought to be the main source of antigen and are defined as rapidly degraded nascent polypeptides due to their inability to achieve a functional state. Although the DRiP hypothesis was postulated almost two decades ago there are still considerable gaps in the knowledge about antigen processing upstream of proteasomal degradation. Interestingly, a report by Minami et al. (2010) found the chaperone BCL2-associated athanogene 6 (BAG6; also named Scythe or BAT3) to be essential for DRiP degradation and MHC class I cell surface expression (Minami et al., 2010). BAG6 is conserved in higher eukaryotes, ubiquitously expressed, and encoded in the MHC class III locus (Banerji et al., 1990; Ozaki et al., 1999; Wang and Liew, 1994). Even though BAG6 is classified as a chaperone, it has no apparent folding activity (Wang et al., 2011). Together with its binding partners TRC35 and UBL4A it associates with long hydrophobic patches in client proteins and prevents aggregation (Leznicki et al., 2013; Mariappan et al., 2010; Minami et al., 2010; Wang et al., 2011). Therefore, BAG6 has also been described as a "holdase" which keeps its substrate in a soluble state (Wang et al., 2011). Because of its binding properties, BAG6 is especially involved in the biogenesis and degradation of polypeptides carrying hydrophobic domains (Kawahara et al., 2013; Lee and Ye, 2013).

In this study, we revisited the role of BAG6 in MHC class I antigen processing proposed by Minami et al. (2010). However, we found no influence of BAG6 knockdown on MHC class I cell surface expression. Moreover, BAG6 was not transcriptionally regulated after stimulation with IFN-γ and presentation of virus-derived peptides was not altered in BAG6 knockdown cells. Together, these results suggest that BAG6 is dispensable or redundant for antigen processing and presentation on MHC class I.

2. Materials and methods

2.1. Mice, viruses, cell lines, and cytokines

C57BL/6 mice (H-2^b), BALB/c mice (H-2^d) and AAD mice (H-2^b, transgenic for an HLA-A*0201/H-$2D^d$ chimeric protein (Newberg et al., 1996)) were originally purchased from Charles River. Mice were kept in a specific pathogen-free facility and used at 8–12

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weeks of age. Animal experiments were approved by the review board of Regierungspräsidium Freiburg.

LCMV-WE was originally obtained from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Vaccinia virus Western Reserve strain (VV-WR) was originally obtained from H. Hengartner (University Hospital Zürich, Switzerland). Recombinant vaccinia virus (rVV) expressing tyrosinase (rVV-Tyr) was a kind gift from V. H. Engelhard (University of Virginia, VA, USA). Both vaccinia virus strains were propagated on BSC-40 cells.

B8-D^b (H-2^d) is a murine fibroblast cell line stably transfected with H-$2D^b$ (Basler et al., 2004). B8-D^b cells were cultured in IMDM. Primary murine embryonic fibroblasts from AAD mice, HeLa cells, HEK293 cells, and MelJuSo cells were maintained in DMEM. The human lung fibroblast cell line IMRS was maintained in MEM including 15% FCS. MelJuSo cells and IMRS cells were a kind gift from N. Koch (University of Bonn, Germany). All media were purchased from Invitrogen-Life Technologies and contained GlutaMAX, 10% FCS and 100 U/ml penicillin/streptomycin.

Recombinant human and murine interferon-γ (IFN-γ) was purchased from Peprotech (Hamburg, Germany) and used at 200 U/ml for two days.

2.2. Transfections

Knockdown of BAG6 was performed through simultaneous transfection of four different siRNAs targeting BAG6. At 80% confluence, cells were transfected with mouse or human BAG6 siRNA (ON-TARGETplus SMART pool siRNA, Thermo Scientific) using DharmaFECT 1 transfection reagent (Thermo Scientific) according to the manufacturer's instruction. After 48 h, cells were transfected a second time to achieve maximum efficiency. Control cells were transfected with a mix of four non-targeting siRNAs (ON-TARGETplus Non-Targeting pool siRNA, Thermo Scientific).

For transient overexpression of BAG6, HeLa cells were transfected with a pCMV6-Entry expression plasmid encoding BAG6-Myc-DDK (OriGene Technologies). Control cells were transfected with empty vector. Transfection was performed at 80% confluence using FuGENE HD transfection reagent (Promega) according to the manufacturer's instructions. Cells were further analyzed 24 h post transfection. Transient overexpression of tyrosinase (Tyr) in HEK293 cells was achieved likewise using a pCMV6-Entry expression construct encoding tyrosinase-Myc-Flag and Trans-IT-LT1 transfection reagent (Mirus Bio LLC.) according to the manufacturer's instructions.

2.3. Flow cytometry

MHC class I surface expression was analyzed by flow cytometry using fluorescently-labeled antibodies. Cells were incubated together with antibodies diluted 1:150 in PBS, 2% (v/v) FCS, 2 mM EDTA for 20 min at 4 °C. Cells were washed and acquired with the use of the Accuri C6 flow cytometer system (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). Anti-H-$2D^b$ FITC (clone KH95), anti-H-$2D^d$ PE (clone 43-2-12) and anti-HLA-ABC APC (clone G46-2.6) antibodies were obtained from BD Biosciences.

2.4. Real-time RT-PCR

Total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Extracted RNA was reverse-transcribed using the Reverse Transcription System (Promega) according to the manufacturer's instruction. Quantitative real-time RT-PCR was performed with the LightCycler Instrument II (Roche) and LightCycler Fast-Start DNA Master SYBR Green I kit (Roche). Gene expression was calculated relative to control cells and normalized to HPRT (hypoxanthine guanine phosphoribosyltransferase) expression using REST-384O software version 2 (Gene Quantification). Sense and antisense primers used for amplification were as follows: BAG6 (human) TACAATAACAATCACGAGGGCC, GGTGGTGTAGTGAGACATAGG; TRC53 (human) CGTGACCTTTGTGTCCAGAG, TTAAACAGAGAAACTGTAGCACGG; HPRT (human) TGGACAGGACTGAACGTCTTG, CCAGCAGGTCAGCAAAGAATTTA; LMP7 (human) AATGCAGGCTGTACTATCTGCG, TGCAGCAGGTCACTGACATCTG.

2.5. SDS PAGE and western blot

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 1% (v/v) Triton X-100, 0.5% (v/v) sodiumdesoxycholate, 0.1% (w/v) SDS) including protease inhibitors (cOmplete EDTA-free, Roche) for 30 min on ice. Lysates were centrifuged at 20,000 × *g* and 4 °C for 15 min. Supernatants were mixed with SDS sample buffer and boiled for 5 min at 95 °C. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane (Whatman). Membranes were blocked for 1 h in Roti-Block solution (Roth) followed by overnight incubation in primary antibodies at 4 °C. Membranes were washed and incubated for 2 h with appropriate peroxidase-conjugated secondary antibodies (Dako). Membranes were washed and proteins were visualized with enhanced chemiluminescence. Primary antibodies used: Rabbit anti-BAG6 (kind gift from R. S. Hegde, Cambridge, UK), mouse anti-α-tubulin (clone AA13, Sigma) and mouse anti-FLAG (clone M2, Sigma).

2.6. Generation of epitope-specific cytotoxic T lymphocyte (CTL) lines

LCMV-specific CTL lines were generated from female C57BL/6 or BALB/c mice infected i.v. with 200 PFU LCMV-WE. Four weeks post infection splenocytes from infected mice were cultured in IMDM 10% FCS, P/S, supplemented with 40 U/ml IL-2, 100 μM 2-mercaptoethanol and 10^{-6} M peptide. Cytokine-supplemented medium was added every other day for 8–14 days. Before CTLs were used in presentation assays, dead cells were removed by Ficoll density centrifugation.

Tyr-specific CTL-lines were generated from male AAD mice infected i.p. with 2×10^6 PFU rVV-Tyr. Splenocytes from memory mice were cultured for three weeks as described above. To increase percentage of specific T cells, CTLs were restimulated twice with peptide-loaded, irradiated (20 Gy) AAD splenocytes on day 8 and 16.

2.7. Synthetic peptides

The synthetic peptides GP276-286 (SGVENPGGYCL), NP118-126 (RPQASGYVM) and NP396-404 (FQPQNGQFI) were obtained from P. Henklein (Charité, Berlin, Germany). The synthetic peptide Tyr369-377 (YMDGTMSQV) was obtained from Sigma.

2.8. Antigen presentation assay

B8-D^b cells or AAD mouse embryonic fibroblasts (MEFs) were transfected with BAG6 siRNA as described above. Transfected cells were harvested and infected with LCMV or rVV with a multiplicity of infection (MOI) of 10 for 2 h, 4 h, or 5 h. Serial dilution of infected cells was performed to achieve different effector to stimulator ratios (E/S). Epitope-specific CTL lines were added to infected cells and incubated in presence of 10 μg/ml brefeldin A for 3 h at 37 °C. Activation of CTLs was determined by intracellular IFN-γ staining and flow cytometry. All samples were prepared as duplicates.