Immunobiology 219 (2014) 924–931

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Hsp90–peptide complexes stimulate antigen presentation through the class II pathway after binding scavenger receptor SREC-I☆

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ARTICLE INFO

Article history:
Received 1 May 2014
Received in revised form 31 July 2014
Accepted 3 August 2014
Available online 10 August 2014

Keywords:
Hsp90
SREC-I
Ova
Antigen presentation
Dendritic cell

ABSTRACT

Molecular chaperones such as heat shock protein 90 (Hsp90) have been shown to form complexes with tumor antigens and can be used to prepare anticancer vaccines largely due to this property. Earlier studies had suggested that mice immunized with a molecular chaperone-based vaccine derived from tumors became immune to further vaccination and that both $CD8^+$ and $CD4^+$ T cells were activated by the chaperone vaccine in a manner dependent on scavenger receptor SREC-I. Here we have investigated mechanisms whereby SREC-I might facilitate uptake of Hsp90-conjugated peptides by APC into the MHC class II pathway for presentation to $CD4^+$ T cells. Our studies showed that antigenic peptides associated with Hsp90 were taken up into the class II pathway by a mechanism dependent on SREC-I binding and internalization and presented to $CD4^+$ T cells. In addition our studies showed that SREC-I could associate with MHC class II molecules on the cell surface and in intracellular endosomes, suggesting a mechanism involving facilitated uptake of peptides into the MHC class II pathway. These studies in addition to our earlier findings showed SREC-I to play a primary role in chaperone-associated antigen uptake both through cross priming of MHC class I molecules and entry into the class II pathway.

Introduction

Molecular chaperones such as heat shock protein 90 (Hsp90) have been shown to form complexes with tumor antigens and have thus been used in preparing anticancer vaccines, largely due to this property (Kunisawa and Shastri, 2006; Murshid et al., 2011a,b). HSPs therefore could function as antigen carriers and it has been suggested that they might use this property to mediate antigen presentation in target APC by facilitating endocytosis of bound polypeptides (Murshid et al., 2010; Srivastava, 2000; Murshid et al., 2012). APCs such as dendritic cells (DC) were shown to take up external antigens and process such polypeptides for presentation to T cells through two distinct pathways including: (1) the classical MHC class II pathway and (2) cross presentation through the MHC class I pathway. Soluble antigens have been shown previously to be partitioned between these two alternative pathways by interacting with different cell surface receptors in DC such as CLEC9, DC-SIGN, DEC205, and the mannose receptor 1 (Murshid et al., 2012; Bonifaz et al., 2002; Burgdorf and Kurts, 2008). Triage between the two pathways thus occurs at the cell surface through the dedicated antigen-binding receptors. It was, however, not clear whether HSP-chaperoned antigens adhered to these mechanisms for selective entry into pathways of antigen presentation. Previous studies showed that HSP–peptide complexes were internalized by a distinct group of receptors compared to free polypeptides (Theriault et al., 2006). These receptors were from the scavenger receptor families and included LOX-1, SREC-I/SCARF-I, and FEEL1/stabilin-1. Both SREC-I and LOX-1 have been shown to mediate the cross presentation of molecular chaperone-bound antigens and lead to activation of $CD8^+$ T lymphocytes (Theriault et al., 2006; Delneste et al., 2002; Murshid et al., 2011a,b). In addition, earlier studies showed that mice immunized with a molecular chaperone-based vaccine derived from tumors became immune to further tumor inoculation and that both $CD8^+$ and $CD4^+$ T cells were activated in a SREC-I dependent manner by the chaperone vaccines (Gong et al., 2010; Gong et al., 2009).

We have set out here to determine whether SREC-I can directly mediate entry of Hsp90-bound antigens into the MHC class II pathway and stimulation of $CD4^+$ T cells.

☆ This work was supported by NIH research grants RO-1CA047407, R01CA119045 and RO-1CA094397.

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http://dx.doi.org/10.1016/j.imbio.2014.08.001
0171-2985/

Materials and methods

Mice

Mice, B6C3F1 (H-2^{bxk}) used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were kept in a specific pathogen-free mouse facility. Studies were done according to institutional guidelines for animal use and care.

Reagents and Abs

Ova was purchased from Sigma–Aldrich (St. Louis, MO). PL19 peptide was custom synthesized (AnaSpec, Fremont, CA) and purified to 95% by HPLC. Rabbit polyclonal human anti-SREC-I Ab was custom synthesized by GenScript (Piscataway, NJ) against specific peptide sequence (TQGTQGSTLDPAGQC). Commercially available anti-SREC-I Ab was purchased from Atlas Antibodies. Anti-MycII Ab was from Sigma. Rat anti-mouse IFN-γ was from BD Pharmingen (San Diego, CA). GM-CSF was purchased from PeproTech (Rocky Hill, NJ). Cy3-transferrin and all other secondary fluorescent Abs were from Jackson ImmunoResearch Laboratories. Alexa 555-labeling kit was from Life Sciences. *Clostridium difficile* toxin B was purchased from Calbiochem. Anti-pMHCII mAb L243 was purchased from BD Biosciences. CIITA plasmid was from Addgene.

Cells and culture conditions

BMDCs were generated from the femur and tibiae of, B6C3F1 mice. The bone marrow was flushed out and cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS and 40 ng/mL GM-CSF for 6 days. On day 3, one-third of the medium was replaced by fresh growth medium. On day 6, the immature BMDCs were used for experiment. Wild-type COS-7 and Hek293 cells were transfected with human full-length SREC-I in pcDNA3 for stable expression of SREC-I. Both cell lines were maintained in Ham's F12 K medium supplemented with 10% heat-inactivated FBS. For generation of stable SREC-I-expressing cell lines, cells were selected and maintained in the same medium plus 400 mg/mL G418. Hek293 and Raw 264.7 cell lines were maintained in DMEM supplemented with 10% heat-inactivated FBS. KG-1 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS.

Plasmids

The pcDNA3.1-SREC-I (human) was a generous gift from Dr. H. Adachi. FLAG–SREC-I construct was made in 3xFLAG-CMV vector. MHCII molecules were gifts from Dr. R. Germain of NIH.

Hsp90–peptide/protein complex and anti–SREC-I-Ab–Ova complex formation

Ova was dialyzed for 36 h at 4 °C with several changes of the buffer (PBS) to remove degradation products as well as possible contaminating peptides from the solution. Small peptides, PL19 (PDEVSGLEQLESIINFEKL) were loaded onto Hsp90 in solution at a molar ratio of 50:1 in PBS, incubated for 10 min at 45–50 °C, and cooled at room temperature for 30–40 min. For Hsp90–Ova conjugate preparation, soluble Hsp90 and excess Ova (1:2 ratio) were mixed and incubated for 10 min at 45 °C. The solution mixtures were then incubated at room temperature for 30 min. Free Ova was removed using Microcon YM-100 (Millipore, Bedford, MA) with a 100-kDa cutoff. Alexa 488- and Alexa 555-labeled Hsp90–peptide/Ova (Hsp90–PC) conjugates were made according to the manufacturer's protocol (Invitrogen). For anti-SREC-I Ab–Ova preparation, Ova was coupled to anti-SREC-I Ab using bis-(sulfosuccinimidyl) suberate as described by the manufacturer (Thermo Fisher Scientific, Waltham, MA) and labeled with Alexa fluorochromes as described for Hsp90 and purified using Microcon YM-100 ultrafiltration (Millipore, Billerica, MA).

Immunofluorescence and microscopic analysis of Hsp90 internalization

COS-7 cells were labeled with Alexa fluor-conjugated Hsp90 complexes for 20–30 min on ice. The ice-cold medium was then replaced by warm medium and incubated at 37 °C for different periods. Cells were later washed with ice-cold stripping buffer (50 mM sodium citrate and 280 mM sucrose [pH 4.6]) to remove unbound Hsp90–PC. Later, the cells were fixed with 4% para-formaldehyde and permeabilized with 0.1% Triton X-100. Cells were stained with different Abs and later analyzed using a Zeiss 510 confocal microscope (Carl Zeiss, Jena, Germany). For analysis of binding to BMDCs, FcRs were pre-blocked. For blocking FcR-mediated nonspecific binding, immature BMDCs were treated with anti-FcγR Ab (CD16/32 specific for FcγRIII, FcγRII) for 10 min at 4 °C at a concentration of 1 mg/mL/million cells. To prevent complication of experiments by Hsp90 interaction with HSP receptor LOX-1, BMDCs were also treated with goat anti-mouse LOX-1 Ab (10 mg/mL) for 10–15 min on ice. Fluorophores were visualized using the following filter sets: 488 nm excitation and band pass 505–530 emission filter for Alexa 488; 543 nm excitation and band pass 560–615 for Cy3; and 633 excitation and long pass 650 for Cy5. Images were taken using a 633 numerical aperture 1.4 oil immersion objective lens (Carl Zeiss, Jena, Germany). Figures were made using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) with little or no contrast adjustments without altering original images.

In vitro cross-presentation assay

Immature BMDCs from mice were plated in 96-well plates post purification. Cells were then pulsed with 10 mg/mL Hsp90 and its Ova conjugates (Hsp90–PL19/Ova), 10 mg/mL free Ova, and also with 10 mg/mL anti-SREC-I–Ova complexes for 5 min to 2 h. In all Ag cross-presentation experiments, a preparation of the BMDC was pulsed with 100 ng/mL PL19 as positive control. For inhibitors and drug treatment, cells were incubated with the drugs before they were pulsed with different Ag preparations. The ligands were then removed and cells fixed with para-formaldehyde for 10 min at room temperature. Later, peptide-specific T cell hybridoma (KZO, a generous gift from Dr. N. Shastri, Department of Molecular and Cellular Biology, University of California, Berklely, CA) was added to the fixed cells at 37 °C for 20 h.

Western blotting and Coimmuoprecipitation assay

HeLa cells were transfected with FLAG-SREC-I and CIITA-Myc for 22 h. Cells were then incubated with or without Hsp90–Ova for 30 mins. Cells were then washed with ice-cold Dulbecco's phosphate buffered saline (PBS) and lysed with NP-40 lysis buffer (containing 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1× HALT protease, and phosphatase inhibitor cocktail, Thermo Scientific). For immunoprecipitation, 1 mg of cell extract was incubated with 5 μg of anti-FLAG antibody (M2) for 2 h at 4 °C followed by incubation with 20 μL of protein A (50% slurry, GE healthcare) plus sepharose beads for overnight at 4 °C. The beads were then washed with NP-40 lysis buffer and complexes were eluted by boiling in Laemmle sample buffer. For western blotting, immunoprecipitated samples were resolved by 4–15% gradient SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were immunoblotted with anti-HLA-DR antibody and later secondary antibody that are HRP conjugated.