Molecular Immunology 55 (2013) 393–399

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

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

Structural and functional mosaic nature of MHC class I molecules in their peptide-free form

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ARTICLE INFO

Article history:
Received 18 January 2013
Received in revised form 14 March 2013
Accepted 15 March 2013
Available online 8 April 2013

Keywords:
MHC class I
Peptide-free form
β_2 microglobulin
NMR
NK cell receptors
Refolding

ABSTRACT

Despite well-organized peptide-loading mechanisms within the endoplasmic reticulum, major histocompatibility complex class I (MHC-I) molecules can be displayed on cell surfaces in peptide-free forms. Although these empty MHC-I (eMHC-I) molecules are presumably involved in physiological and pathological processes, little is known about their structures and functions due to their instability. Using bacterially expressed HLA-Cw*07:02 heavy chain and β_2 microglobulin molecules, we successfully established an *in vitro* refolding method to prepare eMHC-I molecules in sufficient quantities for detailed structural analyses. NMR spectroscopy in conjunction with subunit-specific ^{15}N-labeling techniques revealed that the peptide-binding domains and the adjacent regions were unstructured in the peptide-free form, while the remaining regions maintained their structural integrity. Consistent with our spectroscopic data, the eMHC-I complex could interact with leukocyte Ig-like receptor B1, but not with killer cell Ig-like receptor 2DL3. Thus, eMHC-I molecules have a mosaic nature in terms of their three-dimensional structure and binding to immunologically relevant molecules.

1. Introduction

Major histocompatibility complex class I (MHC-I) molecules are key proteins in the adaptive immune system as they bind endogenous antigenic peptides in the endoplasmic reticulum (ER) and subsequently present these to CD8$^+$ T lymphocytes at their cell surfaces (Saunders and van Endert, 2011). The maturation of MHC-I molecules is initiated by the formation of hetero-dimeric complexes of membrane-bound heavy chains (HCs) consisting of α1, α2, and α3 domains and a light chain, β_2 microglobulin (β_2m). The subsequent peptide loading onto MHC-I molecules within the ER consists of multiple steps involving the molecular chaperone calreticulin, the thiol oxidoreductase Erp57, the peptide transporter associated with antigen processing (TAP), and the type I transmembrane glycoprotein tapasin (Paulsson and Wang, 2003; Wearsch and Cresswell, 2008).

Thus, these ER luminal proteins form peptide-loading complexes that capture empty MHC-I (eMHC-I) molecules, thereby stabilizing their states in order to bind endogenously processed peptides transported by TAP. Peptide-loaded MHC-I (pMHC-I) molecules are released and translocated *via* the Golgi apparatus to cell surfaces where they interact with T cell receptors on the membranes of MHC-I-restricted CD8$^+$ T lymphocytes and natural killer (NK) cell receptors, including leukocyte Ig-like receptors (LILRs) and killer cell Ig-like receptors (KIRs).

Despite these peptide-loading mechanisms within the ER, eMHC-I molecules can be displayed on cell surfaces under certain conditions and are possibly involved in some biological functions (Ortiz-Navarrete and Hämmerling, 1991; Theodossis, 2013). For

Abbreviations: β_2m, $\beta_2$microglobulin; CD, circular dichroism; eMHC-I, empty MHC-I; ER, endoplasmic reticulumn; GSH, glutathione; GSSG, glutathione disulfide; HC, heavy chain; HSQC, heteronuclear single-quantum coherence; KIRs, killer cell Ig-like receptors; LILRs, leukocyte Ig-like receptors; mAb, monoclonal antibody; MHC-I, major histocompatibility complex class I; NK, natural killer; NMR, nuclear magnetic resonance; pMHC-I, peptide-loaded MHC-I; SPR, surface plasmon resonance; TAP, transporter associated with antigen processing; TROSY, transverse relaxation-optimized spectroscopy.

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http://dx.doi.org/10.1016/j.molimm.2013.03.014

example, the NK inhibitory receptor Ly49C reportedly induced inhibitory signals upon interactions with eMHC-I molecules (Benoit et al., 2005). eMHC-I molecules were first identified on the surfaces of the TAP-deficient cell line RMA-S at low temperature (Ljunggren et al., 1990). eMHC-I molecules could bind exogenously added antigen peptides and subsequently underwent conformational transitions coupled with their stabilization (Elliott et al., 1991; Fahnestock et al., 1992; Schumacher et al., 1990). Furthermore, increasing evidence indicates that proliferating lymphoid cells exhibit a heterogeneous pool of MHC-I HCs at their cell surfaces that lack peptides and/or β_2m molecules. These are derived from fully mature pMHC-I molecules and are involved in both *cis* and *trans* interactions with various receptor molecules, including those for insulin, epidermal growth factor, and interleukin 2 (Arosa et al., 2007).

The three-dimensional structures of MHC-I molecules have been extensively studied in their peptide-loaded states (Bjorkman et al., 1987; Madden et al., 1991; Rudolph et al., 2006). However, only a few studies have attempted to elucidate the open conformations of eMHC-I (or HC) molecules, which included the non-classical MHC-I molecules HFE and M10 (Lebrón et al., 1998; Olson et al., 2005). Bouvier and Wiley characterized the peptide-free structures of MHC-I molecules with HLA-B7 HCs (B*0702) by circular dichroism (CD) spectroscopy, fluorescence enhancement of hydrophobic probe, and limited proteolysis; these results indicated a molten globule-like nature of eMHC-I molecules (Bouvier and Wiley, 1998). Peptide-induced structural changes have also been characterized using several conformation-specific monoclonal antibodies (mAbs) (Hansen et al., 2005). In particular, mAb 64-3-7 that is specific for eMHC-I molecules defined a conformational change bearing its epitope located at a conserved 3_{10}-helix-containing region of the α1 domain (residues 46–52), which is close to the N-terminus of the peptide in a pMHC-I molecule and predicted to be exposed to solvent in the peptide-free state (Mage et al., 2012). To date, the atomic details for eMHC-I molecules have been provided by molecular dynamics simulations, which indicate conformational mobility of the α1/α2 domains in eMHC-I molecules, particularly in the region that accommodates the peptide C-terminus (Zacharias and Springer, 2004).

In this study, we attempted to experimentally characterize eMHC-I conformations in solution using NMR spectroscopy. For this purpose, we established an *in vitro* refolding protocol to prepare recombinant eMHC-I complex from bacterially expressed HLA-Cw*07:02 HC and β_2m. The structures of the MHC-I complex in solution were compared between peptide-free and -bound forms based on NMR spectroscopic data and binding activity with immune cell surface receptors.

2. Materials and methods

2.1. Protein expression

β_2m and the extracellular region of HLA-Cw*07:02 HC (residues 1–277) were each overexpressed in *Escherichia coli* BL21(DE3)-CodonPlus cells as inclusion bodies, which were solubilized using 6 M guanidinium chloride. Uniform and selective $^{13}C/^{15}N$-labeling methods were applied according to previously described protocols (Nishida et al., 2006; Yagi-Utsumi et al., 2011). To produce perdeuterated β_2m proteins, cells were grown in M9 minimal medium containing [^{15}N]NH_4Cl (1 g/L), uniformly $^{2}H/^{13}C$-labeled glucose (2 g/L), and $^{2}H_2O$. Solubilized β_2m was refolded by dialysis against 50 mM Tris–HCl buffer (pH 8.0) containing 1 M urea and 1 mM GSH/0.1 mM GSSG for 24 h at 16 °C, then against milli-Q water, and finally lyophilized. The DS11 nonapeptide (RYRPGTVAL) (Falk et al., 1993) was chemically synthesized by a solid phase method.

2.2. Reconstitution of pMHC-I molecules

The HC was denatured in 6 M guanidinium chloride (pH 2.2) for 30 min at room temperature, centrifuged to remove insoluble materials, and the resulting supernatant was slowly diluted 100-fold in ice-chilled reconstitution buffer consisting of 100 mM Tris–HCl (pH 8.0) that contained 400 mM L-arginine, 2 mM EDTA, 5 mM GSH, 0.5 mM GSSG, 10 μM DS11, and 2 μM refolded β_2m. After incubation for 48 h at 16 °C, the protein solution was concentrated with a Centriprep (Milipore YM-3), followed by gel filtration chromatography using a HiLoad 26/600 Superdex75pg column (GE Healthcare Life Sciences) equilibrated with 50 mM Tris–HCl (pH 8.0) at a flow rate of 1.2 ml/min. Purity of the refolding product was checked by SDS-PAGE.

2.3. Reconstitution of eMHC-I molecules

To produce the correctly folded eMHC-I complex in high yields, we prepared it from pMHC-I. The pMHC-I complex was denatured in 50 mM Tris–HCl (pH 8.0) containing 6 M guanidinium chloride for 30 min at room temperature, followed by gel filtration chromatography using an eluent consisting of 50 mM Tris–HCl (pH 8.0) and 6 M guanidinium chloride to isolate the HC with the correct disulfide bonds. Isolated HC was slowly diluted 50-fold in reconstitution buffer consisting of 400 mM L-arginine, 100 mM Tris–HCl (pH 8.0), 2 mM EDTA, and 3 μM β_2m, followed by incubation for 24 h at 16 °C. eMHC-I complex was also purified by gel filtration as described above. Complete removal of the DS11 peptide from pMHC-I molecules was confirmed by reverse-phase HPLC using a Vydac C4 column (Grace Davison Discovery Sciences).

2.4. Surface plasmon resonance analysis

The extracellular domains (residues 1–200) of KIR2DL3 and the two N-terminal Ig-like domains (residues 1–197) of LILRB1 were prepared as described previously (Shiroishi 2003; Maenaka; 1999). Surface plasmon resonance (SPR) experiments used a BIAcore2000 system (GE Healthcare Life Sciences). Biotinylated HLA-Cw*07:02 molecules, with and without the DS11 peptide and biotinylated bovine serum albumin as a negative control were immobilized on a research-grade CM5 chip (BIAcore) onto which streptavidin was covalently coupled. Soluble proteins dissolved in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) were injected over the chip at 25 °C. The binding response at each analyte concentration was calculated by subtracting the equilibrium response measured in the control flow cell from the response in the HLA-Cw*07:02 flow cell. These data were analyzed using BIAevaluation 4.1 (GE Healthcare Life Sciences) and Origin 7 (Microcal, Northampton, MA) software. Equilibrium analysis determined K_d values by nonlinear curve fitting of a Langmuir binding isotherm.

2.5. CD and fluorescence measurements

CD spectra of eMHC-I and pMHC-I molecules at protein concentrations of 0.1–0.2 mg/ml in 50 mM Tris–HCl buffer (pH 8.0) were measured at 20 °C in a 1-mm quartz cuvette using a J-720 spectropolarimeter (JASCO, Tokyo). After subtracting a blank curve, the spectral data were presented as mean residue ellipticities. Secondary structure contents were calculated using reference sets determined by Yang et al. (1986). Fluorescence spectra of eMHC-I and pMHC-I molecules at protein concentrations of 0.01 mg/ml in 50 mM Tris–HCl buffer (pH 8.0) were measured using an FP-777 spectrofluorometer (JASCO) at 25 °C with an excitation wavelength of 280 nm.