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Expression, refolding and crystallization of murine MHC class I H-2D^b in complex with human β_2-microglobulin

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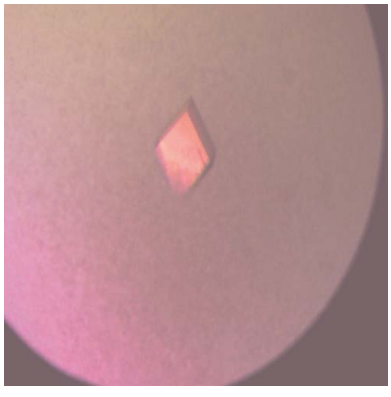

β_2-Microglobulin (β_2m) is non-covalently linked to the major histocompatibility (MHC) class I heavy chain and interacts with CD8 and Ly49 receptors. Murine MHC class I can bind human β_2m (hβ_2m) and such hybrid molecules are often used in structural and functional studies. The replacement of mouse β_2m (mβ_2m) by hβ_2m has important functional consequences for MHC class I complex stability and specificity, but the structural basis for this is unknown. To investigate the impact of species-specific β_2m subunits on MHC class I conformation, murine MHC class I H-2D^b in complex with hβ_2m and the peptide gp33 derived from lymphocytic choriomeningitis virus (LCMV) has been expressed, refolded *in vitro* and crystallized. Crystals containing two complexes per asymmetric unit and belonging to the space group $P2_1$, with unit-cell parameters a = 68.1, b = 65.2, c = 101.9 Å, β = 102.4°, were obtained.

1. Introduction

Class I major histocompatibility complex (MHC) molecules transport peptides derived from intracellular proteins to the cell surface and present them to CD8$^+$ T cells and natural killer (NK) cells, respectively. The MHC class I complex consists of a polymorphic membrane-anchored heavy chain, a non-covalently bound light chain β_2-microglobulin (β_2m) and a peptide. β_2m is a relatively conserved protein that displays 69% identity at the amino-acid level between mouse and human (Gates *et al.*, 1981), allowing cross-species association of β_2m with the MHC class I heavy chain (Bernabeu *et al.*, 1984; Kubota, 1984).

The impact of changes in β_2m species on the stability as well as the function of MHC class I molecules in the context of recognition by antibodies, T cells and NK cells have been probed in a number of studies (Matsumoto *et al.*, 2001; Michaelsson *et al.*, 2001; Shields *et al.*, 1999; Wang *et al.*, 2002; Mitsuki *et al.*, 2004). The interactions between the three constituents of MHC class I molecules are interdependent, so that peptide binding is affected by the β_2m subunit and, conversely, the binding of β_2m to the heavy chain is influenced by the peptide (Shields, Kubota *et al.*, 1998; Parker *et al.*, 1992; Pedersen *et al.*, 1994, 1995). Consequently, β_2m has an impact on both the stability and the conformation of MHC class I complexes, as well as on peptide-exchange capacity (Pedersen *et al.*, 1994, 1995; Shields *et al.*, 1999; Smith *et al.*, 1993; Schultz *et al.*, 1998). It has been previously demonstrated that human β_2m (hβ_2m) in complex with mouse MHC class I heavy chains enhances the stability of the complex and its peptide-exchange capacity compared with mouse β_2m (mβ_2m) (Shields, Moffat *et al.*, 1998; Pedersen *et al.*, 1995). However, the underlying structural mechanisms have not yet been determined. An understanding of these mechanisms would facilitate the development of mutated β_2m that would result in better stabilization of peptide binding.

During the last decade, a large number of inhibitory MHC class I-specific receptors expressed by NK cells and subsets of T cells have been characterized. Using soluble tetrameric MHC constructs, we and others have demonstrated that H-2D^b binds to both NK receptors Ly49A and Ly49C (Hanke *et al.*, 1999; Michaelsson *et al.*, 2000) and that such an interaction is strongly affected by the β_2m subunit (Dam *et al.*, 2003; Michaelsson *et al.*, 2001; Matsumoto *et al.*, 2001; Mitsuki *et al.*, 2004; Wang *et al.*, 2002). Murine MHC class I molecules

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.75–2.7 Å).

X-ray source	I711
Wavelength (Å)	1.029
Resolution (Å)	25.0–2.7
Space group	$P2_1$
Unit-cell parameters	a = 68.1, b = 65.2, c = 101.9, α = 90, β = 102.4, γ = 90
Total No. of unique reflections	22481
No. of observed reflections	82092
Completeness (%)	91.8 (51.4)
$R_{\rm merge}$	4.9 (31.5)
$\langle I/\sigma(I)\rangle$	20.2 (2.4)
Mosaicity (°)	0.77

† $R_{\rm merge} = \sum\sum|I_{hkl} - I_{hkl}(j)|/\sum I_{hkl}$, where $I_{hkl}(j)$ is the observed intensity and I_{hkl} is the average value of the intensity.

in complex with hβ_2m are poorly recognized by these Ly49 receptors in binding and functional studies (Matsumoto *et al.*, 2001; Michaelsson *et al.*, 2001).

The molecular and mechanistic basis underlying the capacity of the co-receptor CD8 to enhance T-cell recognition is still not fully understood. There is a general correlation between T-cell stimulation and the affinity of the T-cell receptor (TCR) for the MHC–peptide complex and the properties of the bound peptides affect activation of the TCR (Holler & Kranz, 2003). It is known that CD8 enhances T-cell antigen recognition by binding to the α_2, α_3 and β_2m domains of MHC class I molecules during recognition of peptide-MHC class I antigens on the surface of target cells (Holler & Kranz, 2003; Gao *et al.*, 1997; Kern *et al.*, 1998; Liu *et al.*, 2003; Luescher *et al.*, 1995). Murine $CD8_{\alpha\alpha}$ makes contact with at least four residues in mβ_2m, but the contribution of these residues to CD8 binding has not been fully investigated.

To investigate the impact of species-specific β_2m subunits on MHC class I conformation in relation to the aspects reviewed above, we will compare the crystal structures of complexes of H-2D^b and gp33, a peptide derived from lymphocytic choriomeningitis virus (LCMV), with mβ_2m on one hand and hβ_2m on the other. The structures of H-2D^b–hβ_2m–gp33 and of the previously solved H-2D^b–mβ_2m–gp33 complexes (Velloso *et al.*, 2004; Achour *et al.*, 2002) only differ in the β_2m subunit. Such comparison should thus allow a more accurate identification of the structural differences imposed by substituting one β_2m subunit for another.

2. Materials and methods

2.1. Generation of soluble H-2D^b–hβ_2m–gp33 MHC complexes

The H-2D^b cDNA sequence coding for amino acids 1–276 (GPHSMRYFETAVSRPGLEEPRYISVGYVDNKEFVRFDSDA-ENPRYEPRAPWMEQEGPEYWERETQKAKGQEQWFRVSLR-NLLGYYNQSAGGSHTLQQMSGCDLGSDWRLLRGYLQFAY-EGRDYIALNEDLKTWTAADMAAQITRRKWEQSGAAEHYK-AYLEGECVEWLHRYLKNGNATLLRTDSPKAHVTHHPRSKG-EVTLRCWALGFYPADITLTWQLNGEELTQDMELVETRPAG-DGTFQKWASVVVPLGKEQNYTCRVYHEGLPEPLTLRWEP) was amplified by RT-PCR. The sequenced PCR product was cloned in the pET-17b expression vector (Novagen) and transformed into BL21(DE3)pLysS (Novagen). The human β_2m sequence encoding amino acids 1–99 (IQRTPKIQVYSRHPAENGKSNFLNCYVSG-FHPSDIEVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFT-PTEKDEYACRVNHVTLSQPKIVKWDRDM) cloned in the pHN1 plasmid vector was kindly provided by Dr E. Y. Jones (Oxford Centre for Molecular Science, Oxford, England). An additional methionine residue was inserted at the N-terminal of the hβ_2m recombinant protein. No tags were used in any of the constructs. Peptide gp33 (KAVYNFATM) was from Research Genetics (Huntsville, AL, USA). Protein expression of both H-2D^b heavy chain and hβ_2m was induced by IPTG and the products were purified separately as inclusion bodies using previously described protocols (Achour *et al.*, 1999, 2002). H-2D^b heavy chain and hβ_2m were produced independently from each other. The proteins were extracted from inclusion bodies in 8 *M* urea. The concentrations of the final products were determined spectrophotometrically and the purity of the products was assessed by SDS–PAGE under denaturing conditions (Laemmli, 1970). Most often, we obtained an average of 60–100 mg ml^{-1} H-2D^b or hβ_2m protein per litre of IPTG-induced bacterial culture. All of the three components that form the MHC complex (heavy chain, β_2m and peptide) are required for the proper refolding of MHC class I complexes. Refolding was performed by dilution at 277 K using a molar ratio of heavy chain:β_2m:peptide of 1:2:10 (Garboczi *et al.*, 1992; Reid *et al.*, 1996). The refolding solution comprised 0.4 *M* L-arginine, 20 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 5 m*M* GSH (oxidized glutathione), 0.5 m*M* GSSG (reduced glutathione) and 100 m*M* PMSF. The β_2m subunit can be refolded alone and is very stable. This molecule is commonly added to the refolding solution and used as a preliminary template in order to increase refolded MHC complex ratios. The refolding of the complex was thus induced by the addition of 3 mg refolded hβ_2m to the refolding solution, which was kept cold at 277 K. A combination of 10 mg peptide, 17 mg hβ_2m and 20 mg H-2D^b was added to the solution and stirred for 48 h. The refolding was pulsed four times with H-2D^b heavy chain at intervals of 8 h with 5 mg H-2D^b in urea. After 48 h, the refolding mixture was concentrated using Amicon concentration devices (Millipore). Refolded H-2D^b–hβ_2m–gp33 complexes were purified and buffer-exchanged into 20 m*M* Tris–HCl pH 7.5 by FPLC using a Superdex 75 gel-filtration column (Amersham Biosciences, Uppsala, Sweden). The yield of refolded complexes was in most cases about 10% of the original material.

2.2. Crystallization of H-2D^b in complex with hβ_2m and LCMV-derived gp33

Crystals were obtained in hanging drops by vapour diffusion. Crystal screens (Hampton Research, Laguna Niguel, CA, USA) were used to establish initial crystallization conditions, which were then refined in a finer grid. The best crystals were obtained in 20% PEG 6K, 100 m*M* Tris–HCl pH 8.0 at room temperature. Typically, 2 µl 6 mg ml^{-1} protein solution in 20 m*M* Tris–HCl pH 7.5 was mixed at a 1:1 ratio with the crystallization reservoir solution (20% PEG 6 K, 100 m*M* Tris–HCl pH 8.0).

2.3. Data collection and processing

Data collection was performed under cryogenic conditions (T = 100 K) at beamline I711 in MAX-lab (Lund, Sweden) (λ = 0.9831 Å) using a MAR 345 image plate. X-ray data for the H-2D^b–gp33–hβ_2m complex were collected to 2.7 Å resolution. Crystals were first soaked in a cryoprotectant solution containing 25% glycerol in reservoir solution (20% PEG 6K, 100 m*M* Tris–HCl pH 8.0) before data collection. The diffraction data were processed using the *HKL* program package (Otwinowski, 1993). Data-collection statistics for the data set used in the final refinement are presented in Table 1. Crystals of the H-2D^b–gp33–hβ_2m complexes belong to space group $P2_1$, with unit-cell parameters a = 68.1, b = 65.2, c = 101.9 Å, β = 102.4°.

3. Results and discussion

H-2D^b heavy chain and β_2m were expressed to high levels (80 ± 10 mg l^{-1}) with a final purity greater than 90%. The H-2D^b molecules were refolded in the presence of hβ_2m and peptide. Chromatographic elution profiles were completely reproducible, demonstrating three peaks comprising aggregated high-molecular-weight complexes (peak 1), the refolded complex (peak 2) and uncomplexed β_2m (peak 3) (Fig. 1*a*). The refolding resulted in yields of approximately 12–15% of MHC complexes, which could be purified to homogeneity by FPLC gel filtration (Fig. 1*b*). As complex formation was limited by availability of the heavy chain, there was always an excess of β_2m, as represented by peak 3 (Fig. 1*a*), which was recycled in subsequent refolding procedures.

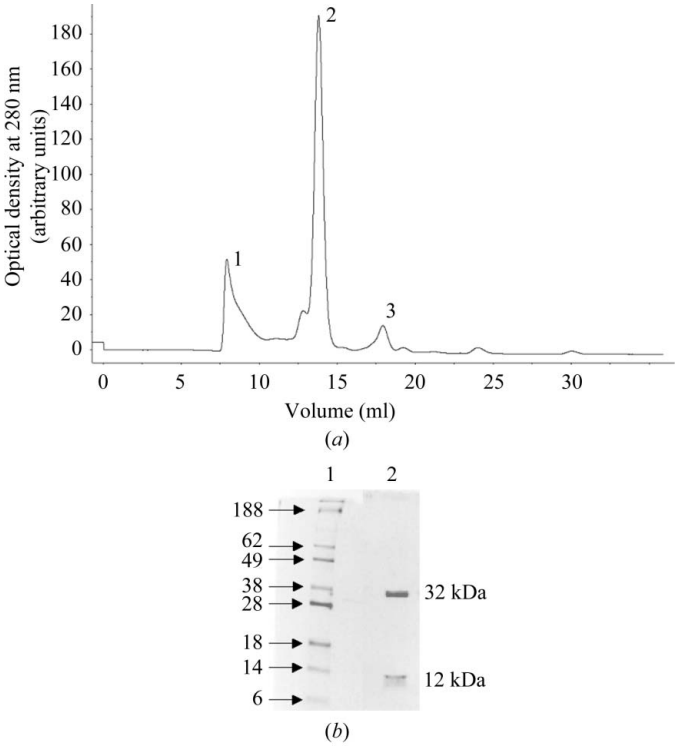

Figure 1
(*a*) Typical chromatogram depicting the elution profile of FPLC Superdex 75 gel-filtrated refolding products. The load rate was 0.5 ml min^{-1}, the sample volume was 200 µl and elution was monitored at 280 nm. The first void peak (1) contains non-native aggregated products, peak 2 represents correctly refolded monomeric H-2D^b complexes and peak 3 represents hβ_2m. (*b*) Proteins separated by SDS–PAGE (15%) under reducing conditions stained with Coomassie brilliant blue. Lane 1, molecular-weight markers (protein size in kDa is indicated to the left); lane 2, purified H-2D^b–hβ_2m–gp33 MHC complex.

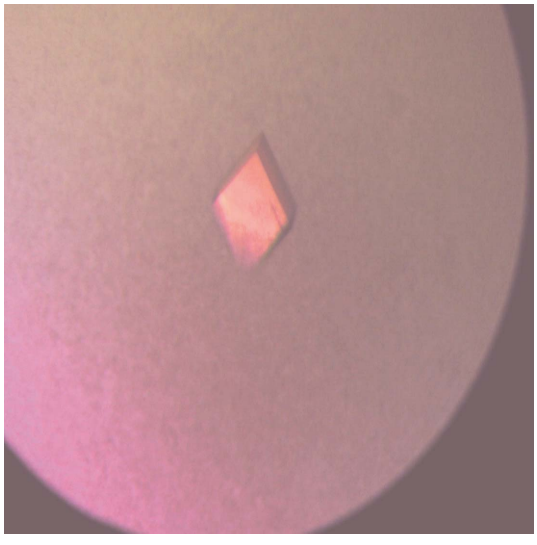

Figure 2
Photograph of a typical crystal of H-2D^b–hβ_2m–gp33. Crystal dimensions are 0.4 × 0.2 × 0.1 mm.

The best crystals appeared after a few days in 20% PEG 6K, 100 m*M* Tris–HCl pH 8.0 at room temperature (Fig. 2). The H-2D^b–hβ_2m–gp33 complex crystallized in space group $P2_1$, with unit-cell parameters a = 68.1, b = 65.2, c = 101.9 Å, β = 102.4°. A Matthews coefficient calculation, determined assuming two MHC complexes per asymmetric unit, resulted in a V_M value of 2.6 Å^3 Da^{-1}, corresponding to 52% solvent content. For three MHC complexes in the asymmetric unit the V_M value was 1.7 Å^3 Da^{-1}, corresponding to only 27% solvent content in the crystal. Thus, the presence of three MHC complexes in the asymmetric unit is not very probable, but cannot be ruled out. A self-rotation analysis showed no evidence of either twofold or threefold non-crystallographic symmetry. However, a native Patterson map revealed the presence of a strong peak (27% of origin peak) at x = 0, y = 0.5 and z = 0.5, implying a pure translational relationship between two complexes in the asymmetric unit. The statistics of the data set are listed in Table 1. The average B factor as derived from a Wilson plot was 74 Å^2. The native data set to 2.7 Å resolution is 91.8% complete and has an R_{merge} of 4.9% and $\langle I/\sigma(I)\rangle$ = 20.2. The structure determination of the H-2D^b–hβ_2m–gp33 complex is currently under way using molecular replacement.

4. Conclusions

We have produced and crystallized mouse MHC class I H-2D^b in complex with human β_2m and the LCMV-derived peptide gp33. Resolution of the structure of this complex combined with the structural comparison with the previously solved crystal structure of H-2D^b–mβ_2m–gp33 (Achour *et al.*, 2002; Tissot *et al.*, 2000) should lead to a better understanding of how the β_2m subunit affects the overall conformation of MHC complexes as well as the stability of the presented peptides. Since these two MHC complexes only differ in the nature of the β_2m species, their comparison should also provide us with important information regarding the conformation of the presented peptide, the role of specific β_2m amino-acid residues in the previously established better stabilization of peptides by hβ_2m and the interaction of MHC class I molecules with T-cell and NK-cell receptors.

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