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Complex assembly, crystallization and preliminary X-ray crystallographic studies of MHC H-2K^d complexed with an HBV-core nonapeptide

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In order to establish a system for structural studies of the murine class I major histocompatibility antigen complex (MHC) H-2K^d, a bacterial expression system and *in vitro* refolding preparation of the complex of H-2K^d with human β_2m and the immunodominant peptide SYVNTNMGL from hepatitis B virus (HBV) core-protein residues 87–95 was employed. The complex (45 kDa) was crystallized; the crystals belong to space group $P222_1$, with unit-cell parameters $a = 89.082$, $b = 110.398$, $c = 47.015$ Å, $\alpha = \beta = \gamma = 90^\circ$. The crystals contain one complex per asymmetric unit and diffract X-rays to at least 2.06 Å resolution. The structure has been solved by molecular replacement and is the first crystal structure of a peptide–H-2K^d complex.

1. Introduction

Major histocompatibility complex (MHC) class I molecules (MHC-I) are plasma-membrane proteins that are expressed by virtually all mammalian cells and play a central role in cellular immune recognition. They present short segments (peptides) of intracellularly processed proteins (forming a peptide–MHC-I complex, abbreviated pMHC-I) to the T-cell receptors (TCR) of cytotoxic T lymphocytes (CTL). This type of specific selective recognition triggers T-cell activation through TCR signal transduction, leading to the CTL cell killing of infected cells (Zinkernagel & Doherty, 1974; Haskins *et al.*, 1984; Townsend *et al.*, 1986; Bjorkman & Parham, 1990), thus conferring cellular immunity against viral infection.

pMHC-I molecules are heterotrimeric structures with (i) a polymorphic membrane-anchored heavy chain with extracellular domains α_1, α_2 and α_3, (ii) a light invariant soluble noncovalently attached β_2-microglobulin (β_2m) unit and (iii) an 8–11-amino-acid peptide positioned in a cleft formed by the α_1 and α_2 domains of the heavy chain (Madden, 1995).

MHC class I genes are characterized by their extraordinary polymorphism, being the most polymorphic genes known to date, which imparts unique spatial and chemical characteristics to each cleft (Trowsdale & Campbell, 1992) and in turn dictates the T-cell epitopes (the binding peptides) of each MHC class I allele. Human HLA-A2 (A*0201) was the first crystal structure of an MHC complex to be determined (Bjorkman *et al.* 1987*a*,*b*) and was soon followed by the structures of murine and human MHC molecules complexed with single peptides (reviewed in Madden, 1995; Jones, 1997). Analysis of these structures improved our comprehension of how cleft architecture affects both peptide presentation and the conformation of the side chains situated on the α_1 and α_2 helices bordering the binding cleft.

The purpose of the present study was to establish a system for structural studies of the murine MHC class I H-2K^d. The complex includes an immunodominant peptide derived from hepatitis B virus (HBV) core protein (amino acids 87–96). The structural analysis of the complex H-2K^d–HBV core 87–96 complex is important for several reasons. Firstly, the structure of H-2K^d is surprisingly unknown, although H-2k^d-restricted peptide motifs, with the anchor being Tyr at position 2 and Ile or Leu at position 9, have been proposed in previous studies (Falk *et al.*, 1991; Maryanski *et al.*, 1993). The H-2K^d molecule has been widely used in functional analysis of T-cell recognition and its epitopes include not only many foreign antigens but also autologous antigens (Amrani *et al.*, 2000; Fan *et al.*, 2000); therefore, analysis of the H-2K^d molecular structure will help in understanding the detail of antigen presentation in cellular immunity and the mechanism of autoimmune disorder. Secondly, HBV is a non-cytopathic DNA virus that chronically infects 350 million people worldwide. Like many other chronic viral diseases and cancers, it is associated with T-cell hyper-responsiveness or tolerance (Chisari, 1995; Chisari & Ferrari, 1995). HBV transgenic mice have already become a model system for the evaluation of immunotherapeutic strategies to break tolerance and terminate persistent HBV infection (Chisari, 1995). Mutation within immunodominant CTL epitopes is closely connected

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with immunological tolerance (McMichael, 1993; Bertoletti *et al.*, 1994). Therefore, structural knowledge of the complex of H-2K^d with HBV core 87–96, which is an immunodominant epitope of HBV major antigen, will be of benefit to research on the structural mechanism of immunological tolerance. Thirdly, we have previously found that a 7-mer peptide (YVNTNMG) of the HBV-core antigen (HBcAg 88–94) is associated with heat-shock protein (HSP) gp96 in liver tissues of patients with HBV-induced hepatocellular carcinoma (HCC; Meng *et al.*, 2001, 2002). This peptide is highly homologous to mouse H-2K^d-restricted 9-mer peptide (SYVNTNMGL; core 87–95). We have also found that this 7-mer binds to H-2K^d *in vitro* (unpublished data), though with a lower affinity than the 9-mer peptide.

We have employed a bacterial expression system and *in vitro* complex assembly to prepare crystals of H-2K^d with the peptide SYVNTNMGL from HBV core-protein residues 87–95. This H-2K^d-restricted peptide has been shown to elicit CTL responses in H-2^d mice with DNA vaccination (Kuhrober *et al.*, 1997). We report here the conditions for successful refolding, purification and crystallization of the complex of H-2K^d with this HBV-core 87–95 epitope. The crystals diffract X-rays to beyond 2.06 Å and the structure has been solved by molecular replacement.

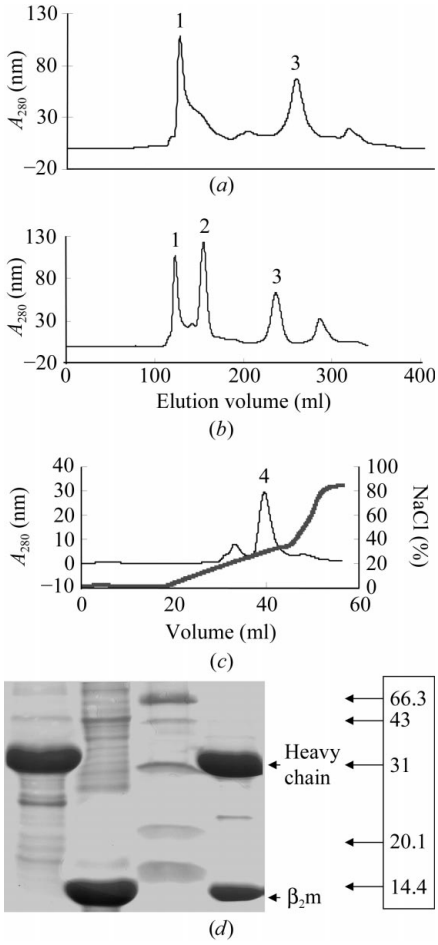

Figure 1
Purification of the complex of H-2K^d with HBV core 87–96 by FPLC Superdex G75 gel-filtration and Mono Q anion-exchange chromatography. (*a*) Refolding attempt without peptide. The first peak represents aggregated heavy chain (labelled 1) and the second peak β_2m (labelled 3). (*b*) Refolding in the presence of peptide (SYVNTNMGL). In comparison with the profile in (*a*), peak 2 represents the correctly refolded H-2K^d complex (45 kDa). (*c*) Further purification of the refolded complex by anion exchange. Peak 4 represents the H-2K^d complex, which was eluted at a NaCl concentration of 17–26 m*M*. (*d*) SDS–PAGE gel (15%) of the purified complex. Lane 1, H-2K^d inclusion bodies; lane 2, β_2m inclusion bodies; lane 3, protein standard markers in kDa; lane 4, the purified refolded H-2K^d complex, showing bands for H-2K^d and β_2m.

2. Materials and methods

2.1. Preparation of H-2K^d and β_2m proteins as inclusion bodies

To construct the expression vector of H-2K^d heavy chain and β_2m, the region encoding amino acids 1–280 of H-2K^d heavy chain and amino acids 1–99 of human β_2m were amplified by PCR and cloned into pET-3a. The expression plasmids were verified by sequencing and transformed into BL21(DE3)pLysS (Novagen). Transformed BL21(DE3)pLysS cells were grown at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} carbenicillin. Isopropyl-D-thiogalactopyranoside (IPTG; Sigma) was added to a final concentration of 0.5 m*M* when the culture reached an OD_{600} of 0.6. After a further 3–4 h incubation at 310 K, the bacteria were harvested and suspended in cold phosphate-buffered saline (PBS) buffer. After being lysed using a sonicator and centrifuged at 20 000*g*, the pellet was washed three times with a solution of 20 m*M* Tris–HCl, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT and 0.5% Triton X-100. H-2K^d and β_2m inclusion bodies constituted most of the pellet.

2.2. Preparation of the H-2K^d complex

This was carried out essentially as previously described by Wiley and coworkers (Garboczi *et al.*, 1992). Briefly, the H-2K^d heavy chain and β_2m inclusion bodies were separately dissolved in a solution of 10 m*M* Tris–HCl pH 8.0 and 8 *M* urea. The synthetically prepared HBV-derived peptide (SYVNTNMGL) was also dissolved in dimethyl sulfoxide (DMSO). H-2K^d heavy chain, β_2m and peptide in a 1:1:3 molar ratio were refolded by dilution. After 24–48 h of incubation at 277 K, the soluble portion was concentrated and then purified by chromatography on a Superdex G-75 (Pharmacia) size-exclusion column followed by Mono Q (Pharmacia) anion-exchange chromatography.

2.3. Crystallization of the H-2K^d complex

The purified complex protein (45 kDa) was dialyzed against crystallization buffer (10 m*M* Tris–HCl pH 8.0, 10 m*M* NaCl) and concentrated to 10 mg ml^{-1}. Initial crystallization conditions were screened using Crystal Screen (Hampton Research). The complex crystallized from conditions containing PEG 20 000. The conditions yielding crystals were further optimized by variation of precipitant and protein concentration and additives. Crystals of good quality can be obtained using 0.1 *M* MES pH 6.5, 18%(*w*/*v*) PEG 20 000, 8%(*v*/*v*) DMSO. Crystallization was performed by the hanging-drop vapour-diffusion method at 291 K. 1 µl protein solution was mixed with 1 µl reservoir solution and the mixture was equilibrated against 200 µl reservoir solution at 291 K.

2.4. Data collection and processing

Data collection from the H-2K^d complex was performed in-house on a Rigaku RU-2000 rotating copper-anode X-ray generator operated at 48 kV and 98 mA (Cu $K\alpha$; λ = 1.5418 Å) with a MAR 345 image-plate detector. The crystals were mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystem with reservoir solution as the cryoprotectant. Data were indexed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

H-2K^d heavy chain could only be refolded in the presence of β_2m and peptide (Figs. 1*a*, 1*b* and 1*c*). The refolding resulted in yields of approximately 10% of complex (45 kDa), which could be purified to homogeneity by Superdex G-75 size-exclusion chromatography and Mono Q (Pharmacia) anion-exchange chromatography (Figs. 1*a*, 1*b* and 1*c*). The chromatographic elution profile showed three peaks corresponding to the refolded complex (45 kDa; peak 2), uncomplexed β_2m (peak 3) and non-native aggregated products (peak 1; Figs. 1*a* and 1*b*). The refolded complex was further purified by Mono Q chromatography and the complex was eluted at an NaCl concentration of 17–26 m*M* (Fig. 1*c*). Moreover, we

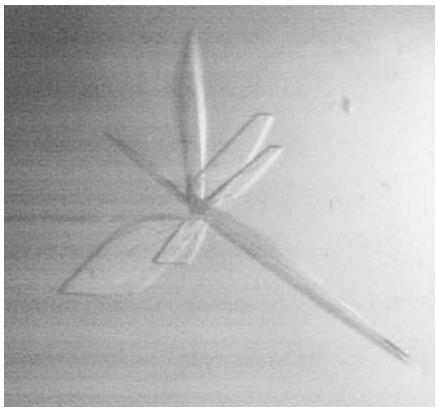

Figure 2
Typical crystals of the H-2K^d–HBV core 87–95 complex, which were used for data collection.

also found that the filtrate of the first refolding solution can be used for further refolding without the addition of peptide if sufficient peptide was added in the first refolding experiment.

Large single crystals (Fig. 2) appeared in 5 d under optimized conditions. The H-2K^d complex crystals belong to space group $P222_1$, with unit-cell parameters a = 89.082, b = 110.398, c = 47.015 Å, $\alpha = \beta = \gamma = 90^\circ$. Assuming the presence of one molecule in the asymmetric unit, the solvent content is calculated to be about 56%. Selected data statistics are shown in Table 1. Structure determination by molecular replacement using the structure of a murine H-2D^b complex (PDB code 1fg2; Tissot *et al.*, 2000) as a search model has been successful and the detailed structure will be reported elsewhere.

Table 1
Data-collection and processing statistics of the H-2K^d complex.

Space group	$P222_1$
Unit-cell parameters (Å)	a = 89.082, b = 110.398, c = 47.015, α = 90, β = 90, γ = 90
Wavelength (Å)	1.5418
Redundancy	7.9 (7.7)
Reflection observed	236416
Unique reflections	29503
Completeness (%)	99.9 (100.0)
$I/\sigma(I)$	28.5 (8.1)
R_{sym} (%)	7.9 (37.9)

† $R_{sym} = \sum_h \sum_i |I_{ih} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$, where $\langle I_h \rangle$ is the mean of the observations I_{ih} of reflection h.

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