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Murine class I major histocompatibility complex H–2D^d: expression, refolding and crystallization

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A truncated soluble form of the murine class I major histocompatibility antigen complex H–2D^d was cloned using an *Escherichia coli* based system. It was expressed, refolded *in vitro* and crystallized in a complex with murine β_2 microglobulin and the peptide RGPGRAFVTI from the V3-loop of the gp160 HIV-1 protein. Crystals belonging to the space group $P2_12_12_1$ with cell dimensions a = 51.3, b = 92.5, c = 108.8 Å were obtained using two different crystallization conditions. The crystals contain one complex per asymmetric unit and diffract to at least 2.4 Å resolution.

1. Introduction

Class I major histocompatibility complex (MHC) molecules are plasma-membrane proteins expressed by virtually all mammalian cells. They transport peptides to the cell surface for presentation to T cells of the immune system. The MHC class I molecules are composed of two subunits: the polymorphic heavy chain that spans the transmembrane bilayer and a lighter invariant noncovalently attached β_2m (murine β_2-microglobulin) unit. The third component of the MHC complex is an 8–11 amino-acid-long peptide positioned in a cleft formed by the α1 and α2 domains of the heavy chain. Recognition of peptides complexed to MHC molecules by T cells is a critical event in initiation of an immune response (Townsend *et al.*, 1986). Furthermore, a novel type of inhibitory receptor on natural killer (NK) cells has been shown to interact with and recognize class I MHC–peptide complexes (for reviews, see Parham, 1997). This inhibitory interaction is fundamental in determining subsequent NK-cell cytotoxicity (Kärre *et al.*, 1986; Ljunggren & Kärre, 1990). Expression of an appropriate MHC class I molecule on the surface of a cell will prevent its destruction by NK cells through an inhibitory effect.

Knowledge of interactions between MHC class I complexes and the T-cell receptor (TCR) derives from functional studies as well as from the three-dimensional structures of soluble MHC class I complexes (Bjorkman *et al.*, 1987; Madden, 1995) and, more recently, of the complex between a TCR and a soluble MHC (Garcia *et al.*, 1996; Garboczi *et al.*, 1996; Housset *et al.*, 1997). Complexes from three different mouse MHC class I loci have been crystallized to date: H–2K^b (Fremont *et al.*, 1992, 1995; Matsumura *et al.*, 1992; Zhang *et al.*, 1992), H–2D^b (Young *et al.*, 1994) and H–2L^d (Balendiran *et al.*, 1997). Analysis of these structures, coupled with peptide binding and functional data, has extended our comprehension of how cleft architecture affects both peptide presentation and the conformation of the side chains situated on the α1 and α2 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 molecule H–2D^d. This is important for understanding how this molecule binds and presents peptides to T cells. The H–2D^d molecule is also central in regulation of NK activity. Ly-49A, the first identified and the best-studied murine NK cell MHC receptor, recognizes H–2D^d but not any of the allelic products that have been crystallized so far (Karlhofer *et al.*, 1992; Kane, 1994; Brennan *et al.*, 1994). A crystal structure of H–2D^d and its comparison with the structure of other MHC class I molecules may provide important clues in the search for possible binding sites for Ly-49 receptors.

We have employed a bacterial expression system and *in vitro* complex formation to prepare crystals of H–2D^d with the peptide RGPGRAFVTI ($P_{RGPGRAFVTI}$) from the V3-loop of the gp160 HIV-1 protein. This peptide sequence may be of particular interest in structural studies since it can bind to four different murine (H–2D^d, H–2D^p, H–2^u and H–2^q; Shirai *et al.*, 1992) and at least one human (HLA–A2; Alexander-Miller *et al.*, 1996) MHC class I molecules. Moreover, there is CTL cross recognition between three of the mouse MHC class I molecules when bound to this peptide (Shirai *et al.*, 1993, 1997). We report here the conditions for successful refolding, purification and crystallization of the H–2D^d:β_2m:$P_{RGPGRAFVTI}$ complex.

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2. Materials and methods

2.1. Preparation of H–2D^d and β_2m

The H–2D^d cDNA sequence coding for amino acids 1–277 was amplified by RT-PCR. The sequenced PCR product was cloned into the pET-3a expression vector (Studier *et al.*, 1990) and transformed into BL21(DE3)pLysS (Novagen). The murine β_2m sequence encoding amino acids 1–99 (cloned by Dr P. Robinson) was provided in a pET-8c plasmid and was a kind gift of Dr P. J. Travers (Birkbeck College, London). The protein production was induced by IPTG and the products were purified as inclusion bodies using slightly modified protocols as described in Garboczi *et al.* (1992) and Reid *et al.* (1996). The concentration of the final products was determined spectrophotometrically and the purity of the product was assessed by SDS–PAGE under denaturing conditions (Laemmli, 1970).

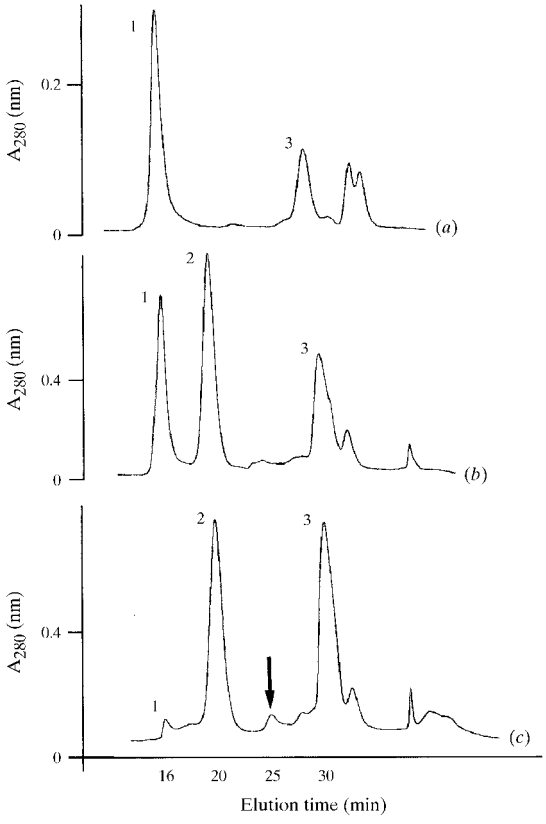

Figure 1
Typical chromatograms depicting the elution profiles of FPLC Superdex 75 gel-filtrated refolding products. Flow rate was 0.5 ml min^{-1}, sample volume was 200 µl and elution was monitored at 280 nm. Each chromatogram derives from an independent experiment; the amount of protein loaded is not directly comparable between them. (*a*) Refolding attempt without peptide. The first void peak represents aggregated heavy chains (1) and the second peak β_2m (3). (*b*) Refolding in the presence of peptide. The first void peak (1) contains non-native aggregated products (70 kDa), peak 2 represents the correctly refolded H–2D^d complex (45 kDa), and peak 3 represents β_2m (12 kDa). (*c*) Improvement of yield through dilution of sample. Peak 1, in which some of the refolded complexes aggregated, has nearly disappeared while the peak with single complexes has increased. The arrow depicts the elution point of heavy-chain run alone.

2.2. Preparation of the H–2D^d complex

Refolding was performed by dilution at 277 K (Garboczi *et al.*, 1992), using a molar ratio of heavy chain:β_2m:peptide of 1:2:10. The refolding of the complex was induced by the addition of 3 mg refolded β_2m to the refolding solution and was pulsed four times with H–2D^d heavy chain as described in Reid *et al.* (1996) at intervals of 8 h with 5 mg of H–2D^d in urea. After 48 h, the refolding mixture was concentrated using Amicon concentration devices. Refolded H–2D^d complexes were purified and buffer exchanged into 20 m*M* Tris–HCl pH 7.0 by FPLC using Superdex 75 gel filtration (Pharmacia). Correct folding of the complex was assessed by Western blotting using an antibody (34-5-8S) which binds a conformation-sensitive epitope present in the peptide-binding region of the H–2D^d molecule (Otten *et al.*, 1992).

2.3. Crystallization of the H–2D^d complex

Crystals were obtained by hanging- and sitting-drop vapour diffusion. Crystal screens (Hampton Research) were used to establish initial crystallization conditions for the H–2D^d complex which were then refined in a finer grid. Typically, 5 µl of a 6 mg ml^{-1} protein solution in 20 m*M* Tris–HCl pH 7.0 was mixed in a 1:1 ratio with the crystallization reservoir solution and allowed to equilibrate at room temperature. Microseeding was used to improve crystal quality and size. Crystals were transferred to a crystallization solution containing 20% glycerol as cryoprotectant before data collection. This transfer was performed in steps of 3%. An SDS–PAGE analysis confirmed the presence of both heavy chain and β_2m in the crystals (data not shown).

2.4. Data collection and processing

Before data collection, the crystals were flash frozen at 100 K in a stream of liquid nitrogen. Data was first collected at beamline X11, EMBL outstation, DESY, Hamburg (λ = 0.91 Å) to 2.6 Å resolution. An additional data set was extended to 2.4 Å resolution at beamline BM14, ESRF, Grenoble (λ = 0.90 Å). In both cases, a MAR Research imaging-plate system was used. One single crystal was used for collection of a full data set. Each frame was collected as a 1.5° oscillation image. The diffraction data were indexed with the program *DENZO* and scaled with *SCALEPACK* (Otwinowski, 1993). The space group was determined with *DENZO*, in combination with the program *PATTERN* (Lu, 1996).

3. Results and discussion

H–2D^d heavy chains and β_2m were expressed to high levels (80–100 mg l^{-1}), with a final purity greater than 90%. The H–2D^d molecules could only be refolded in the presence of β_2m and peptide (Figs. 1*a* and 1*b*), confirming previous studies which reported that peptide is essential for the stability and viability of the complex (Silver *et al.*, 1991). The refolding resulted in yields of approximately 12–15% of complexes which could be purified to homogeneity by FPLC gel filtration. Chromatographic elution profiles were completely reproducible, demonstrating three peaks comprising the refolded complex (peak 2), uncomplexed β_2m (peak 3) and what was thought to be non-native aggregated products (peak 1) (Fig. 1*b*). The size of this first peak, containing aggregates of heavy chain, β_2m and peptide, was reduced by dilution of the sample prior to injection onto the column (Fig. 1*c*). This aggregation peak was thus, at least in part, due to enforced physical aggregation during the final concentration step rather than to a non-native conformation, since a small dilution and resuspension yielded increased refolded product. The yield of uncomplexed β_2m also increased, indicating its release from these physical aggregates along with the desired product. Dilution of the sample in this way allowed maximal production of the correct product required for crystallization (Fig. 1*c*). As complex formation was limited by availability of the heavy chain (no uncomplexed heavy-chain peak was apparent), there was always an excess of β_2m, as represented by the third peak, which was recycled in subsequent refolding procedures.

In the initial screen, the best crystals appeared in 0.1 *M* sodium cacodylate, pH 6.5, 0.2 *M* ammonium sulfate and 30% PEG 8000. Crystals of the same space group and with the same unit cell could also be

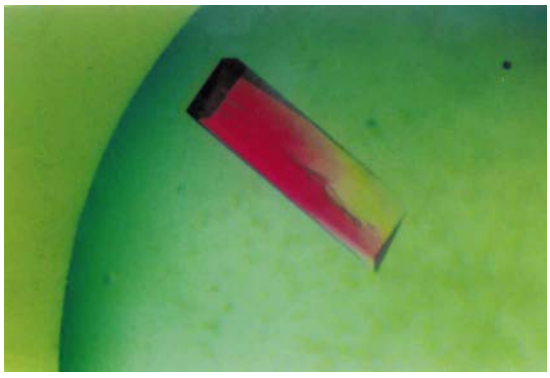

Figure 2
Photograph of a typical crystal of H–2D^d. Crystal size = 0.4 × 0.2 × 0.1 mm.

obtained in 0.1 *M* MES pH 6.0 and 10% PEG 6000. These two crystallization conditions were further optimized by fine screening to 0.1 *M* sodium cacodylate, pH 6.5, 0.15 *M* ammonium sulfate, 15% PEG 8000 or 0.1 *M* MES pH 5.8 and 8% PEG 6000 as the defined optimal conditions. The crystals grown in the cacodylate buffer took a considerably longer time to appear, although larger crystals were usually produced. Thin crystals were used for microseeding to initiate growth in the cacodylate drops. Larger single crystals appeared after about two weeks with a size of approximately 0.4 × 0.2 × 0.1 mm (Fig. 2). The H–2D^d complex crystallized in the space group $P2_12_12_1$ with unit-cell dimensions a = 51.3, b = 92.5, c = 108.8 Å. This corresponds to a V_m = 3.1 Å Da^{-1} (Matthews, 1968) with one molecule per asymmetric unit. The native data set to 2.4 Å resolution is 96.5% complete, has an $R_{\rm merge}$ of 4.0% and $I/\sigma(I)$ = 34 [94.6% completeness, $R_{\rm merge}$ of 29.8% and $I/\sigma(I)$ = 4.7 in the resolution interval 2.46–2.40 Å]. The structure determination of the H–2D^d complex is currently under way using molecular replacement.

4. Conclusions

We have produced and crystallized mouse MHC class I H–2D^d. Non-specific aggregation could be reduced by a simple dilution, thereby further improving the yield of the refolded complex. Resolution of the structure of this complex should lead to further understanding of how MHC–peptide complexes interact with T-cell receptors as well as NK-cell receptors.

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References

Alexander-Miller, M. A., Parker, K. C., Tsukui, T., Pendleton, C. D., Coligan, J. E. & Berzofsky, J. A. (1996). *Int. Immunol.* **8**, 641–649.
Balendiran, G. K., Solheim, J. C., Young, A. C. M., Hansen, T. H., Nathenson, S. G. & Sacchettini, J. C. (1997). *Proc. Natl Acad. Sci. USA*, **94**, 6880–6885.
Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987). *Nature (London)*, **329**, 506–518.
Brennan, J., Mager, D., Jefferies, W. & Takei F. (1994). *J. Exp. Med.* **180**, 2287–2295.
Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A. & Wilson, I. A. (1992). *Science*, **257**, 919–927.
Fremont, D. H., Stura, E. A., Matsumura, M., Peterson, P. A. & Wilson, I. A. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 2479–2483.
Garboczi, D. N., Ghosh, P., Utz, U., Fan, Q. R., Biddison, W. E. & Wiley, D. C. (1996). *Nature (London)*, **384**, 134–141.
Garboczi, D. N., Hung, D. T. & Wiley, D. C. (1992). *Proc. Natl Acad. Sci. USA*, **89**, 3429–3433.
Garcia, K. C., Degano, M., Stanfield, R. L., Brunmark, A., Jackson, M. R., Peterson, P. A., Teyton, L. & Wilson, I. A. (1996). *Science*, **274**, 209–219.
Housset, D., Mazza, G., Grégoire, C., Piras, C., Malissen, B. & Fontecilla-Camps, J. C. (1997). *EMBO J.* **16**, 4205–4216.
Kane, K. P. (1994). *J. Exp. Med.* **179**, 1011–1015.
Karlhofer, F. M., Ribaudo, R. K. & Yokoyama, W. M. (1992). *Nature (London)*, **358**, 66–70.
Kärre, K., Ljunggren, H.-G., Piontek, G. & Kiessling, R. (1986). *Nature (London)*, **319**, 675–678.
Laemmli, U. K. (1970). *Nature (London)*, **27**, 680–685.
Ljunggren, H.-G. & Kärre, K. (1990). *Immunol. Today*, **11**(7), 237–244.
Lu, G. (1996). *Program PATTERN*, http://gamma.mbb.ki.se/~guoguang/pattern.html
Madden, D. R. (1995). *Annu. Rev. Immunol.* **13**, 587–622.
Matsumura, M., Fremont, D. H., Peterson, P. A. & Wilson, I. A. (1992). *Science*, **257**, 927–934.
Matthews, B. W. (1968). *J. Mol. Biol.*, **33**, 491–497.
Otten, G. R., Bikoff, E., Ribaudo, R. K., Kozlowski, S., Margulies, D. H. & Germain, R. N. (1992). *J. Immunol.* **148**, 3723–3732.
Otwinowski, Z. (1993) *Proceedings of the CCP4 Study Weekend: Data Collection & Processing*, edited by L. Sawyer, N. W. Isaacs & S. Bailey, pp. 55–62. Warrington: Daresbury Laboratory.
Parham, P. (1997). Editor. *Immunological Reviews.* Vol. 155. Copenhagen: Munksgaard.
Reid, S. W., Smith, K. J., Jakobsen, B. K., O'Callaghan, C. A., Reyburn, H., Harlos, K., Stuart, D. I., McMichael, A. J., Bell, J. I. & Jones, E. Y. (1996). *FEBS Lett.* **383**, 119–123.
Shirai, M., Kozlowski, S., Margulies, D. H. & Berzofsky, J. A. (1997). *J. Immunol.* **158**, 3181–3188.
Shirai, M., Pendleton, C. D. & Berzofsky, J. A. (1992). *J. Immunol.* **148**, 1657–1667.
Shirai, M., Vacchio, M. S., Hodes, R. J. & Berzofsky, J. A. (1993). *J. Immunol.* **151**, 2283–2295.
Silver, M. L., Parker, K. C. & Wiley, D. C. (1991). *Nature (London)*, **350**, 619–622.
Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). *Methods Enzymol.* **185**, 60–89.
Townsend, A. R., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. & McMichael, A. J. (1986). *Cell*, **44**, 959–968.
Young, A. C. M., Zhang, W., Sacchettini, J. C. & Nathenson, S. G. (1994). *Cell*, **76**, 39–50.
Zhang, W., Young, A. C. M., Imarai, M., Nathenson, S. G. & Sacchettini, J. C. (1992). *Proc. Natl Acad. Sci. USA*, **89**, 8403–8407.