A Soluble Major Histocompatibility Complex Class I Peptide-binding Platform Undergoes a Conformational Change in Response to Peptide Epitopes*

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Elizabeth Rigney, Mayumi Kojima, Ann Gilthorpe, and Tim Elliott‡

From the Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

Class I major histocompatibility complexes (MHC) are heterotrimeric structures comprising heavy chains (HC), β2-microglobulin (β2-m), and short antigenic peptides of 8–10 amino acids. These components assemble in the endoplasmic reticulum and are released to the cell surface only when a peptide of the appropriate length and sequence is incorporated into the structure. The binding of β2-m and peptide to HC is cooperative, and there is indirect evidence that the formation of a stable heterotrimer from an unstable HC:β2-m heterodimer involves a peptide-induced conformational change in the HC. Such a conformational change could ensure both a strong interaction between the three components and also signal the release of stably assembled class I MHC molecules from the endoplasmic reticulum. A peptide-induced conformational change in HC has been demonstrated in cell lysates lacking β2-m to which synthetic peptides were added. Many features of this conformational change suggest that it may be physiologically relevant. In an attempt to study the peptide-induced conformational change in detail we have expressed a soluble, truncated form of the mouse H-2Db HC that contains only the peptide binding domains of the class I molecule. We have shown that this peptide-binding “platform” is relatively stable in physiological buffers and undergoes a conformational change that is detectable with antibodies, in response to synthetic peptides. We also show that the structural features of peptides that induce this conformational change in the platform are the same as those required to observe the conformational change in full-length HC. In this respect, therefore, the HC α1 and α2 domains, which together form the peptide binding site of class I MHC, are able to act independently of the rest of the molecule.

Glycoproteins encoded by the major histocompatibility complex (MHC) class I locus present peptide antigens to cytotoxic T lymphocytes. MHC class I molecules are transmembrane glycoproteins comprising a 45-kDa heavy chain (HC) with three extracellular domains (α1, α2, and α3), noncovalently associated with β2-microglobulin (β2-m). These subunits are assembled in the endoplasmic reticulum (ER) in association with peptide epitopes that are either derived from the cytosol and delivered to the ER by the transporter associated with antigen processing, or generated in the ER itself (see Refs. 1 and 2 for reviews). Biochemical evidence shows that when an appropriate peptide binds to the HC:β2-m heterodimer, the complex is stabilized (3–6) and that, in this respect, peptide binding and class I assembly are linked phenomena in so much as the formation of an MHC class I-peptide complex can be seen as the assembly of a trimolecular complex of HC, β2-m, and peptide. The crystal structures of several MHC class I-peptide complexes have now been solved (reviewed in Ref. 7) and support this view, showing that the peptide ligand is deeply buried in the peptide-binding cleft formed by the α1 and α2 domains. In some cases, as much as 80% of the peptide is buried (8), and in two cases, a salt bridge forms over the bound peptide, between side chains of the α1 and α2 domains on either side of the cleft (9). It is difficult to envisage the diffusion of peptide in and out of this peptide-binding groove according to the classical image of a receptor-ligand interaction. Despite this, several attempts have been made to measure apparent equilibrium binding constants (10) (reviewed in Ref. 11). Also, dissociation rates have been measured for a variety of class I peptide complexes (10). In only one report have both kinetic constants (kₐ and kₐ) and thermodynamic constant (Kₐ) been measured in the same system, and this concluded that peptide-binding groove to which peptides bind is different from those from which they dissociate, the implication being that a conformational change occurs in the class I molecule upon peptide binding (12, 13). Such a conformational change has been observed indirectly in another system. In the absence of bound β2-m, free HC undergoes a conformational change that is detectable with antibodies (4, 14–16), and similarities in the structural requirements for this conformational change and those for stable peptide binding to HC:β2-m heterodimers suggest that this conformational change may be related or identical to that proposed for the HC:β2-m complex upon peptide binding (12, 17).

The relevance of such a conformational change is 2-fold. First, it provides a mechanism for the MHC class I binding site to bind a wide variety of peptide ligands with high affinity. It is more usual to observe receptor ligand interactions in which increased affinity is achieved only at the “expense” of greater specificity. Second, it provides a way in which the MHC class I molecule could signal its release from cofactors that are responsible for retaining incompletely assembled class I molecules in the ER.

As a means of producing MHC class I molecules in a soluble form that could allow a study of the peptide-induced conformational change using high resolution techniques, we chose to make only the portion of a class I molecule that contains the...
peptide binding site. Given the apparent structural independence of this peptide-binding platform, our strategy was to express residues 1–193 of the murine MHC class I molecule H2-D\textsuperscript{b} in *Escherichia coli* and to renature it after solubilization in 8 M urea. We show here the successful expression of this fragment and its ability to undergo a peptide-induced conformational change.

**EXPERIMENTAL PROCEDURES**

**Vectors, Cells, and Antibodies—**pGMT7 is a 3.1-kilobase pair, pGEX-2T-based plasmid containing T7 promoter and terminators flanking a polylinker. *E. coli* strain BL21 (DE3) plys c (is ampicillin, an isopropyl-1-thio-β-D-galactopyranoside-inducible gene for T7 RNA polynase, allowing high level, inducible expression of transfected DNA. The monoclonal antibodies B22.249 and 27-11-13 (4, 18) recognize the native conformation of the H2-D\textsuperscript{b} heavy chain. Rabbit antiserum T18 was raised against D\textsubscript{b}-193 inclusion bodies. It recognizes epitopes on both D\textsuperscript{b} and K\textsuperscript{b} heavy chains, which are lost upon β\textsubscript{2}-m binding but remain when heavy chains bind to peptides in the absence of β\textsubscript{2}-m.

**Cloning and Expression of D\textsubscript{b}-270, and D\textsubscript{b}-193—**Full-length D\textsuperscript{b} heavy chain corresponding to nucleotides 1–874 (amino acids 1–270, D\textsubscript{b}-270), and a fragment of the D\textsuperscript{b} heavy chain corresponding to nucleotides 64–842 (amino acids 1–193, D\textsubscript{b}-193) were amplified by a polymerase chain reaction from cDNA made from the Rasuer-transformed thymoma cell line RMA-S (19, 20). The amplification incorporated a 5′- BamHI and 3′- HindIII recognition site and added nucleotides coding for four additional histidine residues at the C terminus of the fragment. The polymerase chain reaction products were cloned into the vector pGEMT7, and sequenced by the dyeodeoxy method before being transferred into the BL21 strain of *E. coli* for expression. Transformed bacteria were grown in L broth containing 100 μg/ml ampicillin to a density of 0.3–0.5 A\textsubscript{600} units before adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h to induce expression. Clones expressing over 20 mg of protein/ml of culture were isolated.

**Purification and Refolding—**Both D\textsubscript{b}-270 and D\textsubscript{b}-193 were expressed as inclusion bodies and were purified from protein-expressing BL21 transformants as follows. A washed cell pellet was lysed in a minimum volume of 50 mM Tris, pH 8.0, 0.1% sodium deoxycholate, 5 mM EDTA, 2 mM dithiothreitol by sonication (14 min on ice until the decrease in viscosity indicated that all DNA had been sheared). Following cell disruption, and the removal of cell debris by centrifugation, inclusion bodies were pelleted (5 min, 10,000 rpm). Following cell disruption, and the removal of cell debris by centrifugation, inclusion bodies were pelleted (5 min, 10,000 rpm). The polymerase chain reaction products were cloned into the vector pGEMT7, and sequenced by the dyeodeoxy method before being transferred into the BL21 strain of *E. coli* for expression. Transformed bacteria were grown in L broth containing 100 μg/ml ampicillin to a density of 0.3–0.5 A\textsubscript{600} units before adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h to induce expression. Clones expressing over 20 mg of protein/ml of culture were isolated.

**Immunoprecipitation—**1-ml samples of D\textsubscript{b}-193, containing 2–10 μg of protein were incubated at 4 °C with 50 μl of either the H2-D\textsuperscript{b}-restricted H2-Db or K\textsuperscript{b} heavy chain corresponding to nucleotides 1–874 (amino acids 1–270, D\textsubscript{b}-270), and a fragment of the D\textsuperscript{b} heavy chain corresponding to nucleotides 64–842 (amino acids 1–193, D\textsubscript{b}-193) were amplified by a polymerase chain reaction from cDNA made from the Rasuer-transformed thymoma cell line RMA-S (19, 20). The amplification incorporated a 5′- BamHI and 3′- HindIII recognition site and added nucleotides coding for four additional histidine residues at the C terminus of the fragment. The polymerase chain reaction products were cloned into the vector pGEMT7, and sequenced by the dyeodeoxy method before being transferred into the BL21 strain of *E. coli* for expression. Transformed bacteria were grown in L broth containing 100 μg/ml ampicillin to a density of 0.3–0.5 A\textsubscript{600} units before adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h to induce expression. Clones expressing over 20 mg of protein/ml of culture were isolated.

Small, experimental refolding reactions were carried out for analysis by enzyme-linked immunosorbent assay in which 50 μg of D\textsubscript{b}-193 or D\textsubscript{b}-270 inclusion bodies resuspended in 10 μl of 8 M urea were refolded in 2 ml of refolding buffer containing different concentrations of peptide for 48 h at 4 °C. Where appropriate, the refolding buffer also contained 1.36 μM recombinant β\textsubscript{2}-m.

**Immunoprecipitation—**1-ml samples of D\textsubscript{b}-193, containing 2–10 μg of protein were incubated at 4 °C with 50 μl of either the H2-D\textsuperscript{b}-restricted influenza A nucleoprotein epitope residues 366–374 (ASNENMDAM), which is recognized by a redox couple consisting of 0.5 mM oxidized, 5 mM reduced glutathione to encourage appropriate formation of the intramolecular disulfide bond between Cys\textsubscript{101} and Cys\textsubscript{164}. After concentration in a Centriprep C\textsubscript{10} (Amicon), the renatured product was purified by gel filtration on a Superdex 75 FPLC column equilibrated with TBS, using FPLC (Pharmacia Biotech Inc.). Fractions containing soluble, monomeric D\textsubscript{b}-193 were collected, pooled, and concentrated further by membrane filtration. Protein concentrations were determined by the method of Lowry.

Following induction of expression for 5 h, most transformants expressed D\textsubscript{b}-193 as inclusion bodies. Clones expressing greater than 20 mg of protein per liter of culture were isolated and expanded. Fig. 1 (lane a) shows that the crude inclusion body preparation contained a polypeptide of a size corresponding to D\textsubscript{b}-193 (20 kDa) in over 70% abundance. Following washing, greater than 95% of the urea-soluble material was D\textsubscript{b}-193. Refolding, with the replacement of 8 M urea with a stabilizing salt (0.5 M l-arginine), allowed the recovery of around 50% of the input polypeptide in a soluble form. This contained a mixture of products comprising approximately 10% disulfide bonded trimer, 40% disulfide-bonded dimers, and 50% monomer as determined by reducing and nonreducing SDS-polyacrylamide gel electrophoresis, with very high order soluble aggregates (data not shown). Thus, around 5 mg of soluble monomer per liter of culture, was recoverable by gel filtration in TBS, 0.5 M arginine. This was stable at around 1 mg/ml for at least 4 weeks. In the absence of the stabilizing solute, D\textsubscript{b}-193 was slightly less stable and tended to form dimers and trimers when stored at around 0.5 mg/ml at 4 °C for 4 weeks. Routinely, refuruted D\textsubscript{b}-193 was purified and simultaneously transferred into TBS by FPLC gel filtration. A typical chromatogram is shown in Fig. 2. When 50 μM peptide was included in the renaturation, the yield of monomeric D\textsubscript{b}-193 increased

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2 P. Bjorkman, personal communication.
slightly when analyzed by gel filtration, with a corresponding decrease in the yield of dimers and trimers (Fig. 2). In contrast to D₁⁻¹⁹³, full-length heavy chain (Dᵇ⁻²⁷⁰) aggregates completely in the absence of any added peptide during the refolding reaction. This indicates that the formation of low order soluble aggregates is promoted by the presence of the α₂ domain.

**Biological Activity of Dᵇ⁻¹⁹³**—We have previously shown that, in the absence of β₂-m, short peptides that specifically bind to Dᵇ can induce a conformational change in the heavy chain in cell lysates (4, 14, 15). We therefore investigated the ability of Dᵇ-binding peptides to induce a conformational change in Dᵇ⁻¹⁹³ in solution. In TBS (and TBS/arginine), the soluble monomer was unreactive with two monoclonal antibodies that recognize epitopes present in the native conformation of H₂ Dᵇ α₁ and α₂ domains (B22 and 27-11-13). It was, however, recognized by antiserum T18 which was raised to D₁⁻¹⁹³ (see “Experimental Procedures”). Fig. 3 shows that, when either of the Dᵇ binding peptides (FAPGNYPAL or ASNENMDAM) were added to a final concentration of 50 μM, the conformation-sensitive epitope is recovered, and D₁⁻¹⁹³ could now be recognized by mAb B22 249. This is more readily observed for the latter peptide, which has a higher apparent mobility (Fig. 3, c) and strongly suggests that the peptides induce a conformational change in D₁⁻¹⁹³ upon binding. No such conformational change is observed when a control peptide (the Kb-binding peptide SIINFEKL) was added to a similar concentration of 50 μM, the conformation-sensitive epitope is recovered, and D₁⁻¹⁹³ was fractionated on a Superdex 75, FPLC column. The column had previously been calibrated with standard proteins of 158, 44, and 17 kDa (marked). Fractions corresponding to monomeric D₁⁻¹⁹³ were pooled and concentrated.

This is the first description of a soluble fragment of an MHC class I molecule comprising the two N-terminal domains of the protein that form the antigenic peptide-binding site. In the absence of peptides, this 193-amino acid fragment is reason-

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3 T. Elliott, unpublished observation.
ably stable in physiological buffers, but the two domains exist in a non-native conformation as judged by their inability to be recognized by monoclonal antibodies raised against the native whole molecule. A similar situation is seen for full-length heavy chains synthesized in mammalian cells that lack β₂-m. Here, although the immunoglobulin-like α₃ domain appears to be in a native conformation, the α₁ and α₂ domains do not. In detergent lysates, these molecules undergo a conformational change when they bind to antigenic peptides in the absence of β₂-m (4, 14–16), such that the α₁ and α₂ domains acquire epitopes present in the native structure. We have also suggested that peptides bind to the non-native conformation of heavy chain and in doing so, initiate the conformational change (15). This conformational change does not require the presence of the α₃ domain (14), an observation which led us to speculate that it might be possible to observe the same conformational change in the isolated peptide-binding platform formed by the α₁ and α₂ domains. Our results show that this is indeed the case and open up the possibility of studying the conformational change by conventional biophysical techniques. Indeed, D₁₋₁₉₃ᵇ is of a size which is compatible with two-dimensional NMR spectral analysis (22, 23).

We have suggested that the conformational change which we observe for free heavy chain, and now the isolated peptide-binding platform, may be related to a conformational change, which is thought to occur in the heavy chain when peptides bind to the assembled HC:β₂-m heterodimer in vivo. This has been observed indirectly by fluorescence transfer between two MHC class I-bound mAb (17), and by immunoprecipitation of peptide receptive and peptide-bound H₂-Lᵈ molecules with mAb which discriminate between the two forms (24). A more persuasive, but no less indirect, indication of the conformational change arose from a measurement of the kinetic and thermodynamic binding constants of H₂-Dᵇ for Dᵇ-binding peptides (12, 13). This study showed that, for an N-terminally extended peptide ligand, the measured association rate constant corresponded to that predicted from a simple one-step binding model in which \( K_a = k_a / k_d \). However, for the optimal length peptide, the measured association rate is two orders of magnitude higher than that predicted from the \( k_d \) and \( K_a \). This led to the proposal that a conformational change in the class I molecule was responsible for the observed mismatched kinetics of peptide binding, and that only peptides of the optimum length can bring it about. It is interesting to note that the conformational change seen in free heavy chains and D₁₋₁₉₃ᵇ is also observed only in response to optimal length peptides, and that these appear to induce the conformational change rather than simply stabilize the native conformer preferentially (15).

Since the solution of the first MHC class I structure (25), immunologists and structural biologists have been puzzled by the observation that the peptide ligand is so deeply buried in the peptide-binding groove as to be considered part of the MHC class I structure, and have found it difficult to visualize how peptides might diffuse into the binding site as it appears in the crystal structures. This is made all the more difficult when the relatively rapid association rates that have been measured are taken into account. A peptide-induced conformational change from a more “open” or “receptive” peptide-binding groove to the “closed” structure seen by x-ray crystallography would be consistent with these observations. The exact molecular dynamics that constitute this conformational change are entirely unknown at the present time, but a testable model has recently been proposed that involves a movement in the short α₂ helix of the α₂ domain (26).

A peptide-induced conformational change in the MHC class I molecule might also explain why newly assembled HC:β₂-m heterodimers are retained in the endoplasmic reticulum until they become loaded with peptides of an appropriate length and sequence. It is possible that a cofactor in the ER with an

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**Fig. 4.** Quantitative refolding of D₁₋₂₇₀ᵇ and D₁₋₁₉₃ᵇ in the presence of increasing amounts of WT peptide FAPGNYPAL. a, the effect of changing anchor residues at positions 5 (A⁵, FAPGAYPAL) and 5 and 9 (A⁵A⁹, FAPGAYPAAP) on the recovery of refolded HC:β₂-m complexes from refolding reactions containing D₁₋₂₇₀ᵇ and β₂-m is shown in b. The same effect was measured for D₁₋₂₇₀ᵇ and D₁₋₁₉₃ᵇ in the absence of β₂-m (c).
ER-retention signal is able to bind to peptide-receptive but not peptide-loaded MHC class I molecules and that the ability to discriminate between the two forms is due to a peptide-induced conformational change. Indeed, in a nonphysiological experimental system, invariant chain (a cofactor molecule that is normally involved in the biogenesis of MHC class II molecules and not class I) has been shown to bind to H2-Db in a peptide-sensitive manner \((27)\) in vivo. Other, physiologically relevant candidates are the calcium-binding chaperones calnexin and calreticulin \((28, 29)\) and the transporter associated with antigen processing \((30, 31)\).

The production of \(D_1–193\) and the demonstration that in the presence of specific optimal peptides it is recognized by conformation-sensitive mAb, provides, for the first time, a means of studying the peptide-induced conformational change by direct biophysical methods.

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