The Recognition of the Nonclassical Major Histocompatibility Complex (MHC) Class I Molecule, T10, by the γδ T Cell, G8

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Summary

Recent studies have shown that many nonclassical major histocompatibility complex (MHC) (class Ib) molecules have distinct antigen-binding capabilities, including the binding of nonpeptide moieties and the binding of peptides that are different from those bound to classical MHC molecules. Here, we show that one of the H-2T region-encoded molecules, T10, when produced in E. coli, can be folded in vitro with β2-microglobulin (β2m) to form a stable heterodimer in the absence of peptide or nonpeptide moieties. This heterodimer can be recognized by specific antibodies and is stimulatory to the γδ T cell clone, G8. Circular dichroism analysis indicates that T10/β2m has structural features distinct from those of classical MHC class I molecules. These results suggest a new way for MHC-like molecules to adopt a peptide-free structure and to function in the immune system.

Classical MHC class I (class Ia) molecules possess a highly specialized groove occupied by short peptides that are acquired inside the cell during MHC heterodimer assembly. This peptide-MHC interaction not only contributes to the stability of the heterodimer on the cell surface, but forms the basis for its function, as complexes of intracellular pathogen derived peptides with MHC are the ligands for cytolytic cell-mediated immune responses. In this study, we evaluate directly whether components other than the T10/β2m heavy chain and β2m-microglobulin (β2m) are necessary for its recognition and structural stability. We find that E. coli-produced T10 and β2m can be folded in vitro in the absence of peptide or nonpeptide moieties. This is in contrast with classical class I MHC molecules, whose folding of E. coli-produced heavy chain and β2m can take place only in the presence of an appropriate peptide (21, 22). The reconstituted T10/β2m heterodimer...
is biochemically homogeneous and can be recognized by specific antibodies and the G8 γδ T cell. The far-UV circular dichroic (CD) spectrum of T10/β,m is different from that of typical MHC class I molecules. These data suggest that T10 may have evolved to possess distinctive structural features capable of carrying out a specialized function in the immune system.

**Materials and Methods**

Construction of Expression Vectors. The expression cassettes for T10 and T10/L2 (T10/L4) which has the α1 and α2 domains of T10 and α3 domain of the murine class I molecule L4, can be recognized by the Lα3-specific antibody 28.14.8S and G8. This hybrid gene was constructed previously to monitor cell surface expression of T10 in transfected cells in the absence of a T10/T22 heavy chain–specific antibody; reference 15) were constructed using PCR and the oligonucleotide primers GGAATTC

**GCTT**

recognizing L D



**Pharmacia, Uppsala, Sweden** in the presence of 6 M urea.

expression of T10 in transfected cells in the absence of a T10/T22

PCR and the oligonucleotide primers GGAATTC

**TTACC-**

and a stop codon TAA is included in the HindIII oligonucleotide

**ATCTCAGGGTGAGGG** containing the underlined EcoRI, N del, and HindIII restriction sites, respectively. The N del site in the EcoRI–N del oligonucleotide provides an ATG start codon and a stop codon. TAA is included in the HindIII oligonucleotide to terminate the heavy chain following the α3 domain COOH-terminal tryptophan. The PCR was performed with the Ultima

**B121(DE3)pLysS.** Clones containing the heavy chain construct were grown to an OD

**1224**

° of 600 and induced for 2 h with 1 mM IPTG. The harvested cells were resuspended in 10 ml of 25% sucrose, 50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM PM SF, 1 mM DTT, and lysed at 37°C with 1% Triton X-100 and 1 mg/ml lysozyme (Sigma Chem. Co., St. Louis, MO) followed by freeze/thawing. The lysate was incubated for 30 min at 25°C with 30 mM MgCl2, and 30 μg/ml DNase (DN-25; Sigma) followed by the addition of 50 mM EDTA. Inclusion bodies were collected by centrifugation and washed 4–6 times with 20 ml of wash buffer containing 0.5% Triton X-100, 50 mM Tris, pH 8.0, 100 mM NaCl, the inclusion bodies were solubilized in 6 M guanidine–HCl, 100 mM Tris, pH 8.0, 1 mM EDTA. HLA-A2, human β,m (hβ,m), and murine β,m (mβ,m) were expressed and inclusion bodies isolated as described (22, 23). Subunits for the T10/hβ,m folding were size-purified on a Superdex 200 column (Pharmacia, Uppsala, Sweden) in the presence of 6 M urea. Before folding, 0.3 M DTT was added to all subunits.

Folding and Purification of the Heavy Chain–β,m Complexes. Folding of the heterodimer was initiated by a 100-fold dilution of subunits into 1 L of nitrogen-saturated folding solution: 100 mM Tris, pH 8.0, 0.4 M l-arginine, 4 mM oxidized glutathione, 2 mM EDTA, 0.5 mM PM SF for T10/L2/hβ,m or T10/hβ,m and 100 mM Tris, pH 8.2, 25% glycerol, 4 mM oxidized glutathione, 2 mM EDTA, 0.5 mM PM SF for T10/mβ,m. Final protein concentrations were 1 μM T10 heavy chain and 2 μM β,m. Folding reactions were incubated at room temperature for 48 h and concentrated to 30 ml in an Amicon stirred cell (10 Kd cutoff) for fractionation on a Superdex 200 column (Pharmacia). Fractions containing associated T10/L4 heavy chain and hβ,m were identified using a sandwich ELISA. The ELISA-positive fractions for T10/L4 and the corresponding sized fractions for T10/hβ,m or T10/mβ,m were each concentrated ~10 times and subjected to a MonQ™ (Pharmacia) anion exchange column with a linear gradient of 0.1–0.3 M NaCl in 20 mM Tris (pH 7.5). The estimated yields of properly folded T10/hβ,m and T10/mβ,m are 2–4% and 1%, respectively.

The folding of HLA-A2 with HIV pol peptide RT 309-317 (ILKEQVHVGF) was carried out as described (22) with a folding yield routinely ~8%. No heterodimer could be detected when HLA-A2 and β,m were folded in the absence of peptide.

ELISA. The sandwich ELISA for folded T10/L2/hβ,m heterodimer was performed using Immulon IV plates (Dyntec Laboratories, Inc., Chantilly, VA) coated overnight at 4°C with 10 μg/ml 28.14.8S antibody. After a 1-h incubation with analyte at room temperature, a rabbit anti–human β,m polyclonal serum (Boehringer Mannheim, Indianapolis, IN) was added. The assay was developed with a goat anti–rabbit alkaline phosphatase conjugate (Jackson ImmunoRes research Labs, West Grove, PA).

G8 Stimulation Assay. G8 stimulation assays were performed in high binding polystyrene plates (Coster, Cambridge, MA) coated overnight at 4°C with purified T10/β,m complex, moth cytomegocrome c peptide 88–103 loaded I-Ek, or using T10/L4 transfected CHO cells for stimulation of 10⁵ G8 cells per well. Assays were also performed with T10/hβ,m and T10/mβ,m proteins that had been coated overnight at 4°C followed by a 10-h incubation with either PBS containing 2% BSA or RPMI containing 10% FCS at 37°C. The 24-h assay was carried out at 37 or 33°C for T10/hβ,m and T10/mβ,m, respectively. G8 cells express an alkaline phosphatase gene under control of the IL-2 gene NFAT promoter/ enhancer (15). G8 stimulation is measured in fluorescence units, which represent measurements of NFAT-specific alkaline phosphatase activity, using the fluorescent substrate 4-methylumbelliferyl phosphate (Sigma). The dose–response curves are representative of at least three independent experiments.

Circular Dichroism Spectroscopy and Thermal Denaturation Studies. Far-UV CD spectra were recorded in a 0.1 path length cell on an AVIV 60 DS spectropolarimeter (Aviv Associates, Inc., Lakewood, NJ) equipped with a thermoelectric cuvette holder, using a step size of 0.05 nm, a bandwidth of 1 nm, and a time constant of 1 s. Spectra were recorded in sodium phosphate buffer (5 mM, pH 7.0). The spectra show are representative of 3–5 independent measurements (each obtained from five repetitive scans) and were smoothed by the Savitzky–Golay algorithm using a sliding window of 9 (2.25 nm). Far-UVC data is given as (η), the mean residue ellipticity. Thermal denaturation curves were obtained by following the CD signal at 223 nm as a function of the temperature. The temperature was increased in a step-wise mode (2°C intervals) with each temperature jump being followed by a 30-s equilibration time. R ecording time was 100 s. Each point in the melting curves shown in the text represents the average of three independent experiments. R eversibility of the thermal transitions was determined by standard heating/cooling cycles. In each such
cycle, spectra were initially recorded at 25°C and the samples were heated to temperatures above the $T_m$ of the protein complex analyzed and immediately cooled to 25°C. Posttransition spectra were recorded after an equilibration period of 1 h. The CD spectra at high temperatures were recorded separately to avoid the formation of kinetically driven, irreversibly unfolded species due to long incubation times at high temperatures.

Thermodynamic parameters were derived from the CD data presented in the text, assuming a two-state unfolding model. Derivation of the free energy change, $\Delta G$, at physiological temperature (37°C), which lies below the transition region of the unfolding curves where the equilibrium constant, $K$, cannot be directly derived, was made using the following form of the Gibbs-Helmholtz equation: $\Delta G(T) = \Delta H_m(1 - T / T_m) - \Delta C_p(T_m - T) + T \ln \left( \frac{T}{T_m} \right)$, where $T$ is the Kelvin temperature, $T_m$ is the midpoint temperature of the thermal unfolding transition, $\Delta H_m$ is the enthalpy change for unfolding measured at $T_m$, and $\Delta C_p$ is the difference in heat capacity between the folded and unfolded conformations. $T_m$ and $\Delta H_m$ were obtained from the van't Hoff equation: $\Delta H = RT^2(d \ln K) / (dT)$, in which $R$ is the gas constant. $\Delta H_m$ values used to derive the free energy of the two T10 forms were 96 and 85 kcal/mol for the human–mouse and mouse–mouse combinations, respectively. $\Delta C_p$ values were assumed to be independent of temperature (24) and were estimated from the following: $\Delta C_p = (\Delta H / T_m)$, where $\Delta H$ was used in all calculations. Values of $K(T)$ inside the transition region of the unfolding curves were derived from the following relation: $K(T) = (\Theta_N - \Theta_T) / (\Theta_T - \Theta_U)$, where $\Theta_N$ and $\Theta_U$ are the limiting ellipticity values representing the native and unfolded states, respectively, and $\Theta_T$ is the observed ellipticity at $T$. Baseline corrections of the row ellipticity values were made only for data below the transition zone. The end product of the main unfolding transition was represented by a single molar ellipticity value.

Figure 1. Purification of reconstituted T10/β2m heterodimer by ion exchange chromatography. (A) SDS-PAGE analysis of T10 heavy chain (lane 1) and hβ2m (lane 2) in urea, a 0.25 M NaCl ion exchange column peak fraction from the T10/hβ2m purification (lane 3), and a 0.5 M NaCl high salt wash ion exchange column fraction (lane 4). Subunits in lanes 1 and 2 have been size purified in 6 M urea after solubilization in guanidine-HCl. The gel was stained with Coomassie blue. (B) SDS-PAGE analysis of T10 heavy chain (lane 1) and mβ2m (lane 2) solubilized in guanidine-HCl, a 0.27 M NaCl ion exchange column peak fraction from the T10/mβ2m purification (lane 3), and a 0.5 M NaCl high salt wash ion exchange column fraction (lane 4). The gel was stained with Coomassie blue. (C) Ion exchange chromatography profile from the T10/hβ2m heterodimer purification. Two major peaks, one at 0.25 M NaCl in the 0.1–0.3 M NaCl gradient and a second peak eluting at 0.5 M NaCl in the high salt wash, are observed.
Results

E. coli–produced T10 and β2m Subunits Can Be Folded into a Stable Heterodimer in the Absence of Peptide. It was shown previously that T10/T22 protein can be expressed stably on cells lacking a functional peptide-loading mechanism (15, 16, 19). In addition, incubation of T10/Ld-expressing cells with peptide libraries of 8 amino acids in length or shorter does not increase the level of surface T10/Ld expression (Schild, H., M. Jackson, and Y.-h. Chien, unpublished data). These results suggest that T10/T22 may not require peptide binding for stable expression on the cell surface at physiological temperature. The fact that T10/T22 expressed on these peptide loading–deficient cells can stimulate G8 as well as those molecules expressed on normal cells further suggests that a peptide-free form of these molecules is functional. To evaluate definitively whether T10 and β2m without peptide are sufficient for maintaining the structural stability and function of the complex, we expressed both components separately in E. coli, purified and denatured each component, and folded them together in vitro.

To detect properly folded material in the absence of an anti-T10/T22 antibody or a suitable mouse β2m antibody, we first performed folding experiments with a soluble form of the chimeric T10/Ld heavy chain molecule and human β2m. T10/Ld, which has the α1 and α2 domains of T10 and α3 domain of the murine class I molecule Ld, can stimulate G8 and can be recognized by the Ld α3-specific antibody 28.14.8S (15). Human β2m can be recognized by an anti-human β2m polyclonal serum.

Soluble forms of the T10/Ld and T10 heavy chain proteins were produced by truncating the extracellular domains just before the transmembrane region. All proteins (T10, T10/Ld, hβ2m, and mβ2m) were produced as inclusion bodies and, thus, they could be isolated to a high level of purity by standard washing procedures (Fig. 1, A and B). For the folding of T10/hβ2m, subunits were subjected to gel filtration in the presence of 6 M urea to further purify heavy chain and hβ2m away from residual bacterial components (Fig. 1 A). The folding of subunits (T10/Ld with hβ2m, T10 with hβ2m, and T10 with mβ2m) was initiated by dilution of the denatured subunits according to modified published procedures (22, 25). To isolate heterodimer, the folding reactions were concentrated and fractionated by gel filtration chromatography. Fractions containing the renatured T10/Ld/hβ2m were detected by a sandwich ELISA. Corresponding fractions from the T10/hβ2m or T10/mβ2m foldings were combined and the heterodimers were further purified by ion exchange chromatography. In a gradient of 0.1–0.3M NaCl, a major peak eluting at 0.25 M NaCl or at 0.27 M NaCl was observed for the T10/hβ2m and T10/mβ2m, respectively, while the rest of the protein eluted in the 0.5 M NaCl high salt wash (Fig. 1 C; data not shown). In each case, SDS-PAGE indicated that the major peak within the gradient contained both the heavy chain and β2m, whereas fractions from the high salt wash contained heavy chain alone (Fig. 1, A and B).

Fractions from the ion exchange column were assayed for their ability to stimulate G8 γδ T cells. For all heterodimer purifications, only the material eluting within the major peak in the gradient was active in these assays. The folded T10/hβ2m complex was found to stimulate G8 to the same degree as T10/Ld transfected CHO cells (Fig. 2 A), which stimulate G8 to a higher level than the naturally expressing EL4 or PCC3 cells (15). The T10/mβ2m complex was also stimulatory (Fig. 2 B), but at an ∼10-fold lower level.

The lower stimulatory activity of T10/mβ2m in these experiments is most likely due to its lower thermal stability compared with that of the T10/hβ2m form (as discussed below). This could cause T10/mβ2m to be more sensitive to the denaturing effects of the coating process (26). We have preincubated T10/hβ2m and T10/mβ2m with media...
at 22°C for 10 h before T cell stimulation assays. This treatment does not change the dose-response curves for either complex compared with those without preincubation. Together, these results clearly indicate that the complex has been correctly folded and can be recognized by G8, without the addition of peptide or nonpeptide components and, likely, without the contribution of a media or serum component.

T10/β2m Is Structurally Different from Classical MHC Class I Molecules. Far-UV CD spectroscopy has been used to analyze the structure and thermal stability of classical MHC class I molecules and the rat neonatal Fc receptor (FcRn), an MHC-like molecule that functions as an IgG transporter (27–31). To obtain similar parameters for the reconstituted T10/β2m heterodimer, we subjected both T10/β2m and T10/mβ2m to CD analysis (Fig. 3, data not shown). Interestingly, the spectra of both T10/β2m and T10/mβ2m are red-shifted compared with those reported for classical MHC class I molecules and FcRn (27–31). This difference was further verified by comparing the CD spectrum of T10 with that of E. coli-expressed and folded HLA-A2 molecules complexed with HIV pol peptide (Fig. 3). These data suggest that although these molecules are likely to have similar folds, T10 has structural properties distinct from classical class I MHC molecules (32).

The thermal denaturation profile of the T10/β2m complex is shown in Fig. 4 A. At neutral pH, the melting curve reveals two transitions. The first is characterized by a transition temperature midpoint (Tm) of 49°C and reflects the simultaneous dissociation and unfolding of the T10 heavy chain. The second transition, with a Tm at 63–64°C, is characterized by a sign reversal of the CD signal and closely parallels the unfolding profile of free β2m (Tm = 64°C, data not shown; references 29, 30), implying that its denaturation is largely independent of the heavy chain. Consistent with its lower activity in G8 stimulation assays, the T10/mβ2m complex is less stable than the mouse-human combination, with a Tm = 43°C (Fig. 5). Mouse β2m was observed to have a Tm = 62°C (data not shown). For each heterodimer, the thermal transition is largely reversible (see Fig. 4 B; data not shown).

Assuming a standard two-state unfolding model, the free energy change at a particular temperature, ΔG(T), can be estimated from the melting curves shown here. At physiological temperature (37°C), we calculate free energy changes of ~3.3 and 1.5 kcal/mol for the T10/β2m and T10/mβ2m heterodimers, respectively. These values for T10/β2m and T10/mβ2m can be translated into expected ratios of folded to unfolded species of 200:1 and 11:1, respectively, at 37°C. The structural basis for the different thermal stabilities of these heterodimers is presently under investigation.

By comparison, both forms of T10 are less stable than classical class I molecules complexed with an appropriate
peptide, for which free energies >5 kcal/mol and Tm of 65–72°C have been reported (29, 30). However, with the exception of the Kd molecule, MHC class I molecules are critically unstable in the absence of peptide and can not be assembled. On the other hand, the stability of FcRn, which is stimulated by association with a molecule not covalently linked to the complex. However, regardless of the nature of such a stabilizing factor, by substituting the mouse β2m with human β2m in our vi tro system, we were able to increase the stability of the T10 complex. T10/hβ2m can stimulate G8 at levels similar to that of the best stimulator cells. These results indicate that, while an additional factor may be necessary for the stable expression of T10 on the cell surface, it does not confer specificity. Thus, this single feature required for G8 recognition is a properly folded and stable T10 heavy chain and β2m heterodimer.

This scenario is reminiscent of the recognition of murine class II I-Ek by the γδ T cell LBK5. While I-Ek requires peptide for stable expression on the cell surface, the bound peptide does not confer specificity for its recognition by LBK5. As shown previously, LBK5 can recognize I-Ek stabilized by a variety of different peptides (15). Thus, these two examples of γδ T cell recognition differ fundamentally from the recognition by αβ T cells of classical or nonclassical MHC. In the latter case, the peptide or nonpeptide moieties being presented contributes significantly to the specificity of the recognition. However, unlike I-Ek, there is no evidence that T10 binds peptide. This assertion is based on the observation that no peptide(s) other than those derived from the antibodies used for immunoprecipitation can be eluted from T10/Ld molecules isolated from cells (19), as well as our result presented here that the folding of heterodimer does not require peptide. These observations are consistent with the primary amino acid sequences of T10/T22, which suggest that these molecules may lack the necessary structural features to bind peptide.

Most notably, T10/T22 possess a 3-amino acid deletion within the α1 domain and a 12-amino acid deletion within the α2 domain (14). Thus, assuming that T10 and T22 molecules fold similarly to classical MHC molecules, the α2 α-helical region, the conserved COOH-terminal peptide-binding pocket (pocket F), as well as the outermost β strand of the α1 domain and the outermost β strand of the α2 domain, would all be significantly altered (14).

Such structural features in T10 are not shared by FcRn, a class I β2m molecule that does not bind peptide. Crystallographic studies revealed that FcRn is structurally similar to MHC molecules in its conservation of the α1 and α2 domain topology of two helices that span a single β sheet, but the presence of a proline at position 162 introduces a break in the α2 helix of the molecule, causing a shift of its α helices and resulting in a peptide-binding groove filled with side chains (34). Consistent with the crystallographic analysis, it was shown that the CD spectrum of FcRn and classical MHC are very similar (35). However, both spectra are blue-shifted when compared with that of T10/β2m. This observation indicates that T10 is likely to possess structural properties not found in either MHC class I molecules or in FcRn. Thus, these results suggest a new way for an MHC class I-like molecule to adopt a peptide-free structure.

An alternative to the hypothesis that T10/T22 needs an
additional factor for stability is the possibility that rapid turnover is a useful property in a ligand for γδ T cell recognition. There is strong evidence that at least one role for γδ T cells in the immune system is the surveillance for cells that have become stressed or damaged (36–39). T10 molecules expressed on the cell surface are likely to have a shorter half-life than classical MHC molecules. Consistent with this, preliminary antibody stainings indicate low T10 cell surface expression on primary lymphoid cells and EL4 cells (Crowley, M., and Y.-h. Chien, unpublished data). It is arguable that a rapid turnover may better enable T10 to act as a transient target of sensory immune cells.

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