Characterization of an incompletely assembled major histocompatibility class I molecule (H-2K^b) associated with unusually long peptides: Implications for antigen processing and presentation

(β_2 -microglobulin/ β_2 -microglobulin⁺ class I/ β_2 -microglobulin⁻ class I/peptides)

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Contributed by Stanley G. Nathenson, February 1, 1994

ABSTRACT We have identified two forms of a major histocompatibility complex (MHC) class I molecule, H-2K^b, distinguishable by specific antibodies through a study of a genetically engineered mouse cell line that overexpresses these molecules. One form, a complex associated with β_2 -microglobulin (native, β_2 m⁺ class I), is detectable by conformation-dependent antibodies. The other form, which remains after preclearing cell lysates of native class I, is only poorly, if at all, associated with β_2 -microglobulin (β_2 m⁻ class I) and is detectable by an antiserum against the cytoplasmic tail region of H-2K molecules. Both forms are also present in normal cell lines. The affinity-purified native class I molecules bind short peptides (8 or 9 residues) and assemble tightly with β_2 -microglobulin. In striking contrast, the β_2 m⁻ class I molecules bind peptides that are longer (>15 residues) than those bound to native class I molecules. This finding is consistent with the recent evidence that peptides longer than 8–10 amino acid residues are transported into the endoplasmic reticulum and suggests the possibility of a control step for peptide presentation by MHC in which the incompletely processed peptides bind to the heavy chain and a selected fraction undergoes final processing and presentation on the cell surface.

The heavy and light chains of major histocompatibility complex (MHC) class I molecules assemble with short peptides of 8–10 amino acid residues in the endoplasmic reticulum (ER) and only then egress to the cell surface (1–4). When peptides derived from intracellular pathogens are so displayed, they form targets for antigen-specific receptors of cytotoxic T lymphocytes leading to lysis of infected cells and clearing of the pathogen (refs. 2–4 and references therein). The rules for peptide selection, binding, and class I assembly have been established in some detail (see refs. 3–9 for reviews). However, the early steps—e.g., the formation of derived peptides and their transport into the ER—are less clearly understood. Several proteins, some of them encoded in the class II region of the MHC, are thought to play a role in the proteolytic processing of proteins to class I binding peptides and in the transport of such peptides into the ER (10–26). Of these proteins, TAP1 and TAP2 translocate peptides that can associate with class I molecules (22–26). An intriguing finding is that these proteins can also transport peptides longer than the canonical class I binding peptides (23, 25, 26). This suggests that the proteolytic machinery indiscriminately processes proteins to peptides of various lengths and those that are 8 or 9 amino acid residues or longer

are translocated into the ER where they are available to class I heavy and light chains for assembly.

The translocation of peptides longer than 8 or 9 amino acid residues into the ER raises several questions regarding the assembly of class I molecules *in vivo*. How are longer peptides trimmed further in size so that they fit properly in the MHC groove? Does this editing occur before or after the peptide becomes associated with class I molecules? If the longer peptides bind class I molecules, what might be the structural features (conformation) of such complexes and what might be the fate of such class I–long peptide complexes? Here, in studies relevant to these questions, we demonstrate that class I molecules, both in normal cells and in cells engineered to overexpress these molecules, exist in at least two forms distinguishable by specific antibodies. Biochemical analyses of the two forms purified from the overexpressor cells show that one form consists of tight complexes of heavy and light chains with short peptides, while the other form is a complex of longer peptides with heavy chains that may be only weakly associated with the light chain.

MATERIALS AND METHODS

Cell Lines and Antibodies. EL-4 and RMA cells (H-2^b) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, and L-glutamine. The construction and characterization of H-2K^b high expressor cells (Kb-high) will be described in detail elsewhere (S.J. and S.G.N., unpublished data). Monoclonal antibodies (mAbs) mAb74 and mAb90 are I-A^b specific (27) and were kindly provided by K. J. Hasenkrug and J. Stimpfling; Y3 (28, 29) and B22-249 (28) are conformation-dependent H-2K^b- and D^b-specific mAbs, respectively. Anti-X8 antiserum was prepared by immunizing rabbits with keyhole limpet hemocyanin-conjugated H-2K^b exon 8-derived peptide (CKVMVHDDHSLA) as described (30) (R. Spathis, E. C. Goyarts, and S.G.N., unpublished data). Although H-2K^b is overexpressed in NS0 plasmacytoma cells, the endogenous H-2K^d class I molecules are barely detectable by anti-X8 antiserum (data not shown).

Metabolic Labeling with [³⁵S]Methionine and Immunoprecipitation. Approximately 5×10^5 Kb-high or 5×10^6 EL-4

Abbreviations: MHC, major histocompatibility complex; ER, endoplasmic reticulum; mAb, monoclonal antibody; PAS, protein A-Sepharose; β_2 m, β_2 -microglobulin; RP, reversed phase.

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cells were labeled for 60 min with 50 μ Ci of [35 S]methionine (1 Ci = 37 GBq) after a 60-min methionine starvation. Cells were harvested in ice-cold phosphate-buffered saline and the cell pellets were lysed in buffer (50 mM Tris-HCl, pH 8.0/150 mM NaCl/5 mM EDTA) containing 1.0% CHAPS. The postnuclear fraction was precleared overnight with normal mouse serum (NMS) mixed with protein A-Sepharose (PAS) beads at 4°C. The lysates were first incubated at 4°C with 10 μ l of Y3 ascites bound to $\approx$ 50 μ l of PAS for 60 min. After the beads were pelleted, the supernates were incubated at 4°C with 3–5 μ l of anti-X8 antiserum bound to $\approx$ 50 μ l of PAS for 60 min. Immunoprecipitates were washed six times and class I molecules were dissociated by boiling for 5 min in SDS/PAGE loading buffer (31), loaded onto SDS/15% polyacrylamide gels, and detected by autoradiography.

Affinity Purification of Class I Molecules. H-2K^b molecules were affinity purified from detergent lysates of $\approx$ 10 $\times$ 10⁹ Kb-high cells. Affinity columns were prepared as described (32, 33). The lysate was precleared over a NMS-bound PAS column and passed sequentially over a mAb74- and a mAb90-coupled PAS column, followed by four passes over a Y3-coupled PAS column to ensure complete depletion of native K^b molecules. The Y3 cleared lysate was then passed twice over an anti-X8-coupled PAS column. As control for non-specific peptide binding to anti-X8 antiserum, the detergent lysate of cells overexpressing H-2D^b (Db-high) was subjected to affinity chromatography; the lysate was passed sequentially over NMS, mAb74/mAb90, anti-X8 (two times), and B22-249 (three times) columns, in that order. All columns, except the NMS columns, were washed and eluted as described by Joyce *et al.* (33).

Peptide Isolation, Purification, and Characterization. Peptides were eluted from class I molecules and separated from the heavy and light chains by Centricon 10 ultrafiltration as described (33). Approximately 5% of peptides eluted from native K^b and 10% of peptides eluted from β_2 m⁺ K^b and from anti-X8 eluate of Db-high lysates were subjected to pool sequencing by automated Edman degradation with a model 477A sequencer (Applied Biosystems). The remaining ultrafiltrate from β_2 m⁺ and β_2 m[−] K^b (where β_2 m⁺ indicates association with β_2 -microglobulin and β_2 m[−] indicates poor or no association with β_2 -microglobulin), native D^b, and the control peptide preparation from anti-I-A^b columns were loaded onto a C18 column (2.1 $\times$ 250 mm) (Vydac, Hesperia, CA) and separated by reversed-phase HPLC (RP-HPLC) (HP 1090/HP 1040 DAD; Hewlett-Packard) by a modification of the previously described method (33). Peptides were eluted by using a gradient of 0.06% trifluoroacetic acid/5% acetonitrile in water (buffer A) and 0.05% trifluoroacetic acid/80% acetonitrile in water (buffer B): 0% B at 10 min, 37% B at 73 min, 70% B at 105 min, 90% B at 115 min, and 100% B at 120 min established at 0.2 ml·min^{−1}. Chromatographic data were collected at 210 and 280 nm. Five-drop fractions of the eluting peptides were collected and fractions corresponding to peaks were pooled, concentrated, and sequenced.

RESULTS

MHC Class I Molecules Exist in Cells in at Least Two Forms.

One approach to study the structure and function of peptides associated with MHC class I molecules is to characterize the naturally processed peptides associated with these antigen presenting receptors. However, such studies are impeded by the relatively low levels of expression of class I molecules (an average of 5 $\times$ 10⁴ class I molecules per cell) and their ability to bind a large array (on the order of 2000 to >10,000) of structurally related peptides (reviewed in refs. 5 and 6). Thus, an analysis of a large number of the naturally processed peptides would require between 5 and 10 nmol of purified

class I molecules as the starting material (33). Therefore, we developed a genetically engineered mouse cell line (NS0 plasmacytoma cell line; H-2^d) that overexpresses H-2K^b. This cell line, Kb-high, allowed us to purify $\approx$ 5 nmol ($\approx$ 300 μ g) of class I molecules from $\approx$ 10 $\times$ 10⁹ cells (S.J. and S.G.N., unpublished data) when only 5–15 μ g of K^b molecules is obtained from equivalent numbers of C57BL/6 splenocytes (S.J. and D. Roopenian, unpublished data), RMA, and EL-4 cells (S.J., K.K., and S.G.N., unpublished data). A detailed biochemical characterization of Kb-high cell line will be described elsewhere (S.J. and S.G.N., unpublished data).

We find that Kb-high cells express two immunochemically distinct forms of MHC class I molecules that can be distinguished by sequential precipitation with specific antibodies. Y3 (a conformation-dependent mAb) immunoprecipitates from detergent lysates of metabolically radiolabeled Kb-high cells (Fig. 1, lane 2) a form of H-2K^b that is associated with β_2 m (β_2 m⁺ class I). However, subsequent precipitation of the Y3 cleared, labeled lysates with an antiserum raised against a peptide in the cytoplasmic region of H-2K molecules (anti-X8) reveals a second form of class I molecule that is weakly or not at all associated with β_2 m (β_2 m[−], class I) (lane 4). In these cells, this β_2 m[−] K^b is not a result of limited amounts of β_2 m since Kb-high cells contain an excess of light chains (S.J. and S.G.N., unpublished data). Immunochemical analyses of normal cell lines, such as EL-4 (lanes 1 and 3) and RMA (data not shown) cells, also reveal the presence of both β_2 m⁺ and β_2 m[−] forms of class I molecules.

Peptides Longer Than the Canonical Class I Binding Ligands Are Associated with the β_2 m[−] Form of Class I Molecules. The discovery of a pool of class I molecules binding only weakly or not at all to β_2 m raises the question as to the relationship of this form of class I to that of native β_2 m⁺ class I molecules. Therefore, the structural features of β_2 m[−] K^b were explored by biochemical means. These studies were carried out in the Kb-high cells because they express $\approx$ 20-fold greater amounts of β_2 m⁺ and β_2 m[−] K^b molecules than EL-4 (Fig. 1) or RMA

FIG. 1. Immunochemical detection of two distinct forms of H-2K^b molecules. When [35 S]methionine-labeled H-2K^b overexpressor (Kb-high; lanes 2 and 4) or EL-4 (lanes 1 and 3) cell lysates are immunoprecipitated with the conformation-dependent mAb Y3, the native (β_2 m⁺) form of K^b associated with β_2 m (lanes 1 and 2) is detected. Subsequent immunoprecipitation of the Y3 cleared lysate with anti-X8 antiserum (against the cytoplasmic tail of H-2K molecules) identifies the β_2 m[−] form of K^b, which is not associated with β_2 m (lanes 3 and 4). H, H-2Kb heavy chain; L, β_2 m.

Table 1. Assignment of residues from sequencing of peptide pool eluted from native (β_2m^+) K^b molecule

Position in peptide											
1	2	3	4	5	6	7	8	9	10	11	12
V	G	Y	K	F	E	X*	L	V	—†	—	—
I	A	F	Q		T		F				
P		P	E		P		V				
A		L	N		V						
			R		D						
					K						
					G						
					A						

A fraction of the total peptide pool was sequenced up to 12 cycles and the assignments of the residues at given positions are based on the decreasing order of their yields. Residues in boldface type indicate K^b binding anchor residues.

*Significant phenylthiohydantoin-derivatized amino acid yield was not obtained in this cycle.

†Significant phenylthiohydantoin-derivatized amino acid yield was not detectable beyond this cycle.

(data not shown) cells. Peptides associated with the two forms of class I molecules were isolated and a fraction of the peptide pool was subjected to microsequence analysis. These analyses reveal that peptides associated with native (β_2m^+) K^b have features similar to those described previously (34, 35). The peptides are 8 or 9 amino acid residues long and contain the dominant K^b binding anchor residues (34, 35) at positions 3, 5, and 8/9 (P3, P5, and P8/9) (Table 1). Thus, β_2m^+ native K^b molecules are complexed with canonical short peptides with appropriate anchor residue motifs for class I binding.

In striking contrast to the features of peptides eluted from the native β_2m^+ K^b , pool sequencing of an aliquot of the unfractionated peptides associated with β_2m^- K^b molecules (Table 2) reveals that identifiable residues are present up to 15 cycles of Edman degradation, suggesting that some of the peptides are longer than 8 amino acid residues (Table 2). Also in this peptide pool, unlike the native class I binding motif, the expected K^b anchor residues at P3 and P5, phenylalanine and tyrosine, are present from sequencing cycles 3–8 (Table 2). Furthermore, the expected hydrophobic residues at the C terminus of K^b binding peptides, valine and methionine, are not only present at P8 and P9 as for peptides from native class

Table 2. Assignment of residues from sequencing of peptide pool eluted from β_2m^- K^b molecule

Position in peptide														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
P	R	Y	L	L	Q	L	P	V	V	K	V	M	V	V
V	E	F	M	Y	Y	I	F	D	Q	M	M	V		
K	H	G	Y	F	F	S		P	E					
I	A	S	F			Y		R	R					
L						R			Y					
						N								

Peptides eluted from β_2m^- class I were subjected to automated amino acid sequencing up to 15 degradation cycles. In contrast to sequence termination beyond cycle 9 for native class I binding peptides, significant phenylthiohydantoin-derivatized amino acid yields were obtained up to the 15th cycle. The assignment of residues to a position is based on decreasing yields of each amino acid at a given cycle. Tyrosines and phenylalanines, indicated in boldface type, occur from cycle 3 to cycle 8, which is different from that seen in a similar analysis of native class I-associated peptides (see Table 1). Similarly, the hydrophobic residues, also in boldface type, that normally occur at the C terminus of the canonical peptides are seen in significant yields from cycle 8 to cycle 15.

I but are present from sequencing cycles 8 to 15 (Table 2). As a control for nonspecific binding of peptides to anti-X8 antiserum, detergent lysates of cells overexpressing H-2D^b were passed over the anti-X8 column and treated in an identical manner to lysates of Kb-high cells. This preparation did not contain peptides (data not shown). Thus, the peptides associated with the β_2m^- class I molecules are not due to copurification of peptide contaminants during anti-X8 affinity chromatography.

To further characterize the physicochemical nature of the long peptides associated with β_2m^- class I molecules, we have purified the peptide preparations from both β_2m^+ and β_2m^- class I molecules by RP-HPLC (Fig. 2) and subjected a selection of peaks to automated microsequence analysis. Although the RP-HPLC profiles of the two peptide preparations show some peaks eluting at identical times (e.g., see retention time between 38 and 45 min in Fig. 2), the two profiles are clearly distinct (e.g., see retention times between 50 and 57 min and between 78 and 86 min in Fig. 2). Thus, the β_2m^- K^b pool is associated with an array of peptides different from those associated with the β_2m^+ K^b molecules.

FIG. 2. Comparison of RP-HPLC profiles of peptides eluted from β_2m^- (solid line) and β_2m^+ (dashed line) K^b molecules. In the same experiment, as a control for background, peptides eluted from material bound to an anti-I-A^b (not expressed by NS0 cells) mAb column was also chromatographed (data not shown).

Table 3. Amino acid sequence analysis of individual HPLC peaks show different length peptides are associated with β_2m^+ and β_2m^- H-2K^b molecules

K ^b form/ peak ID	Position of residue at peak																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
β_2m^+																				
Y60.2	S	G	P	Q	F	V	Q	L	—	—	—	—	st							
β_2m^-																				
X44*	E	T	S	D	L	S	L	P	D	G	K	<u>V</u>	<u>M</u>	V	H	<u>D</u>	<u>P</u>	H	st	
X80a	P	G	V	K	V	K	P	V	I	Q	K	<u>E</u>	<u>F</u>	V	P	<u>G</u>	<u>L</u>	—	—	—
X80b	A	K	I	A	A	X	H	L	F	G	M	R	X	I	Q	A	K	—	—	—
X50	V	X	F	X	F	M	L	A	N	A	F	P	H	K	D	A	Y	L	F	—
X86	X	V	F	E	F	Q	E	P	M	P	I	X	F	X	G	G	M	E	Y	A

In all the long peptides, a putative H-2K^b binding motif can be identified. In peptide X44, the motif stretches between P6 and P13 or P14; in X80a a nonamer motif is seen from P9 to P17; in X80b an octamer motif between P7 and P14 is seen; in peptide X50 the motif is within a decamer (P9–P18); and the motif in X86 is within the nonamer (P1–P9). st, Sequencing terminated. β_2m^+ H-2K^b binding anchor residues are indicated in boldface type. Possible presence of a second phenylthiohydantoin-derivatized amino acid is indicated by underlining.

*This sequence matched the cytoplasmic tail region of H-2K^b and K^d. At present we do not know whether X44 is specifically or nonspecifically associated with nascent class I. The remaining peptide sequences do not match any protein in the data bank (Swiss-Prot, release 25.0; April 1993).

To gain more precise details on the characteristics of individual peptides bound to the two forms of K^b, amino acid sequence analysis was performed on several selected RP-HPLC purified peaks. A single peptide sequence was obtained on amino acid sequencing of a peak (Y60.2, Fig. 2) associated with native (β_2m^+) K^b molecules. This peptide is 8 amino acid residues long and contains the K^b-specific binding motif (Table 3). On the other hand, amino acid sequences of several individual RP-HPLC peaks of peptides eluted from β_2m^- class I molecules reveal that they are 17 to >20 amino acid residues long (Table 3). Furthermore, analysis of the sequence data did not reveal the presence of the canonical K^b binding motif within them (Table 3). These data confirm that associated with the β_2m^- form of K^b are peptides longer than the canonical class I binding peptides.

DISCUSSION

In the present study we have established that, in cells, MHC class I molecules exist in at least two forms that are serologically and structurally distinct. One form, the native class I molecule, exists in a tight complex with β_2m and short (8 or 9 residues long) peptides and is detectable by a conformation-dependent antibody. The other form, the β_2m^- class I, exists predominantly as a complex of K^b heavy chains with unusually long peptides (>17 residues long), is poorly or not at all associated with β_2m , and cannot be detected by our available conformation-dependent antibodies. This form of class I is detectable by antibodies whose specificity does not require β_2m binding to heavy chains.

The term β_2m^- class I molecule is used as a functional definition for a form of class I in which the light chain, under our experimental conditions, fails to coprecipitate (copurify) with the heavy chains. K^b molecules associated with the unusually long peptides have an altered conformation since these molecules can no longer be recognized by the conformation-dependent mAb Y3 (Fig. 1). This altered conformation—i.e., lack of recognition by the Y3 mAb—may be a direct consequence of the binding of long peptides to the heavy chain. Several groups have demonstrated that peptides, as well as β_2m , independently and synergistically influence the conformation of the heavy chain (36–41). It is conceivable that the long peptide-heavy chain complex has a weak affinity for β_2m , because of which it dissociates from the complex during overnight preclearing of cell lysates, resulting in the loss of Y3 epitope as reported (36, 39).

The discovery of a pool of K^b molecules associated with long peptides and lacking β_2m raises the question of the function of the constituents of this pool. An individual long

peptide-K^b molecule could proceed along two or more possible pathways. One pathway would lead to the formation of the native class I associated with β_2m and short peptides. In this case, the long peptide-associated β_2m^- class I complex has to be converted to the short peptide-associated intermediate and then to native class I by tightly complexing with β_2m . This would appear to depend on various factors such as the presence of a high-affinity binding motif within the long peptide and the presence of trimming proteinase in the ER lumen.

It is possible that the β_2m^- K^b molecules, or at least some of these molecules, are inert complexes and do not follow the above pathway but instead are targeted to a degradative compartment. This second pathway is conceivable for the complexes described here because none of the long peptides sequenced contained a complete K^b binding anchor motif (–n-Tyr/Phe/Pro-Phe/Tyr-hydrophobic residue–n; where *n* represents the remainder of the peptide chain). However, it is possible that in our system the long peptides containing the complete binding motif may have been short-lived, were rapidly converted to short peptides, and would not be recovered in a steady-state sampling as described here. Thus, it is conceivable that both the above processes are ongoing within the cells—i.e., the β_2m^- class I complexes are processed and assembled to form native class I molecules containing the canonical short peptides that egress to the cell surface, while those complexes that cannot be further processed are eventually degraded. While we cannot substantiate a definite function of the long peptide-associated β_2m^- K^b, its presence in cells confirms the previous demonstration that long peptides are transported into the ER (23, 25, 26) and suggest a control step in the presentation of peptides by class I molecules wherein the “best-fitting epitopes” proceed in the MHC processing pathway while the “less satisfactory ones” are degraded.

We thank Y. Shi for technical help and advice with amino acid sequencing and data analysis; R. Spathis and Dr. E. C. Goyarts for the preparation and initial characterization of anti-X8 antiserum; Drs. B. Bershtein, H. L. Ploegh, and M. D. Scharff for critical evaluation of the data and helpful suggestions on the manuscript; and R. Spata for secretarial assistance. This work was supported by a Cancer Research Institute Postdoctoral Fellowship (S.J.) and National Institutes of Health Grants AI07289, AI33184, and CA13330 to S.G.N.

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