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Different MHC Class I Heavy Chains Compete with Each Other for Folding Independently of $\beta_2$-Microglobulin and Peptide

Sophie Tourdot,* Mohamed Nejmeddine,* Simon J. Powis, † and Keith G. Gould3*

We reported previously that different MHC class I molecules can compete with each other for cell surface expression in $F_2$ hybrid and MHC class I transgenic mice. In this study, we show that the competition also occurs in transfected cell lines, and investigate the mechanism. Cell surface expression of an endogenous class I molecule in Chinese hamster ovary (CHO) cells was strongly down-regulated when the mouse Kd class I H chain was introduced by transfection. The competition occurred only after Kd protein translation, not at the level of RNA, and localization studies of a CHO class I-GFP fusion showed that the presence of Kd caused retention of the hamster class I molecule in the endoplasmic reticulum. The competition was not for $\beta_2$-microglobulin, because a single chain version of Kd that included mouse $\beta_2$-microglobulin also had a similar effect. The competition was not for association with TAP and loading with pepti

d, because a mutant form of the Kd class I H chain, not able to associate with TAP, caused the same down-regulation of hamster class I expression. Moreover, Kd expression led to a similar level of competition in TAP2-negative CHO cells. Competition for cell surface expression was also found between different mouse class I H chains in transfected mouse cells, and this competition prevented association of the H chain with $\beta_2$-microglobulin. These unexpected new findings show that different class I H chains compete with each other at an early stage of the intracellular assembly pathway, independently of $\beta_2$-microglobulin and peptide. The Journal of Immunology, 2005, 174: 925–933.

Major histocompatibility complex class I molecules present short, intracellularly derived peptides at the cell surface for possible recognition by CTL and NK cells. For each MHC class I molecule expressed on the plasma membrane, the highly polymorphic H chain is assembled with the conserved L chain $\beta_2$-microglobulin ($\beta_2m$) and peptide in the endoplasmic reticulum (ER) in a complex series of events that has been studied extensively and reviewed in detail (1, 2). With the characterization of the aminopeptidase that trims peptides in the ER (3–5), and the recent discovery of the importance of peptide trimming in the cytoplasm (6), the different stages and components of the class I assembly pathway now appear to have been identified.

An important aspect of the class I Ag presentation pathway that is sometimes neglected is the fact that human or murine cells express up to six different classical MHC class I alleles. These different class I molecules are often assumed to function independently of each other within cells, although it is known that they can interact with components of the assembly pathway in different ways (7, 8) and have different rates of trafficking to the cell surface (9, 10) and different levels of cell surface expression (11). Moreover, it has been known for a long time from mouse experiments that the presence of a particular MHC class I molecule can profoundly alter a CTL response restricted by another class I molecule (12). This is also the case for influenza A virus-specific CTL responses in humans (13), showing that the particular combination of class I alleles present is important, not only the MHC class I molecule presenting the specific peptide. The mechanisms responsible for these effects are still not fully understood.

The presence of multiple class I H chains in a single cell gives rise to the possibility that they compete with each other, either for $\beta_2m$ or for peptide, or for some aspect of the intracellular assembly process, such as peptide loading and optimization. Indeed, two specific examples of competition between MHC class I molecules under special circumstances have been reported (14, 15). Influenza A virus nucleoprotein contains two different epitopes that overlap by 8 aa residues and are presented by different HLA class I molecules. These two class I molecules appear to compete for a common peptide fragment in the ER (14). Secondly, competition between class I molecules for access to TAP has been shown in a rat system (15). In contrast to human and mouse, the rat has two groups of functionally different TAP2 alleles. One group of alleles encodes transporters that are restrictive in their specificity, and are unable to supply peptides for the efficient assembly of certain rat class I molecules. Under these circumstances, the class I molecules are persistently retained in the ER in association with TAP, and competitively reduce the association of other, different rat class I molecules with TAP (15). However, in both of these two types of competition, there was no effect on the intracellular trafficking or steady state level of cell surface expression of the class I molecules subject to competition (14, 15). In contrast, reducing the level of $\beta_2m$ expression in mice by reduction of the gene copy number did

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¶Abbreviations used in this paper: $\beta_2m$, $\beta_2$-microglobulin; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; HA, hemagglutinin.
decrease class I cell surface expression, and in a differential manner, such that some class I molecules were affected to a greater extent than others (16). The results implied that when β2m is limiting, each mouse class I molecule competes for β2m with a different efficiency, leading to different levels of cell surface expression of the respective molecules (16). However, the significance of this type of competition at physiological levels of β2m is not known, and the effects of other, nonclassical MHC class I molecules that associate with β2m need to be considered.

Previously, using F1 hybrid and MHC class I transgenic mice expressing normal levels of β2m, we have shown that different class I molecules can indeed compete with each other for cell surface expression, and that this may have functional effects and significantly alter CTL responses against influenza virus (17). We have also found that the same type of competition between class I molecules occurs in transfected cell lines, and the aim of this study was to identify the stage of the intracellular assembly and trafficking of MHC class I molecules at which this competition takes place.

Materials and Methods

Antibodies

Anti-MHC class I H-2K^k FITC-conjugated Ab was purchased from Sero- tec. Anti-H-2K^k FITC, anti-H-2D^d PE, and anti-mouse Ig PE Abs were from IgG and the anti-mouse IgG1 was from Molecular Probes. Anti-rat Ig FITC was purchased from Sigma- Aldrich. The hybridoma M1/42 (rat anti-mouse MHC class I) was purchased from the American Type Culture Collection. The hybridoma H37-kd (rat pan anti-mouse MHC class I) was purified from T. Elliott (University of Southampton). Alexa Fluor 568-conjugated goat anti-mouse IgG1 was from Molecular Probes.

Cell cultures and transfection

Chinese hamster ovary (CHO) cells (K1 subline), L929, and BW5147-SP3 cells (SP3) (19) were all obtained from the Sir William Dunn School of Pathology, University of Oxford. T-Rex-CHO cells used for tetracycline-inducible expression were purchased from Invitrogen Life Technologies. TAP2-negative CHO cells (20) were kindly supplied by N. Shastri (University of California). All CHO cell lines were cultured in Nutrient Mixture F-12 (HAM) with 4 mM glutamine and 5% FCS; medium for T-Rex-CHO cells was used, according to the manufacturer's instructions, using the oligodeoxynucleotides 5'-GGGCTCTGATGGCCTGGCCGCGGTGGGCGCGGTGGTGTCACG-3' and 5'-AA-3' for the Kd H chain. This fragment was cloned into the expression vector pPG5 (described above), and its complete sequence was verified by automated DNA sequencing (Genetics Core Facility, Imperial College). The cDNA clone encoding the HA of influenza virus A/PR/8/34 (Mount Sinai strain) has been described before (23), and was cloned into the ToPO TA cloning kit (Invitrogen Life Technologies), and the insert was checked by automated DNA sequencing (Genetics Core Facility, Imperial College). The hybridoma M1/42 (rat pan anti-mouse MHC class I) was purified from T. Elliott (University of Southampton). Rabbit anti-exon 8 anti- serotonin (recognizing exon 8 in the cytoplasmic domain of Kd H chains) (18) was obtained from S. Drisch (University of Duesseldorf). Rabbit anti-exon 3 anti-serotonin (recognizing exon 3 in the cytoplasmic domain of Kd H chains) was generated as a gift of A. Townsend (University of Oxford). Rabbit anti-H-2K d FITC, anti-H-2D b PE, and anti-mouse Ig PE Abs were used, according to the manufacturer's optimized protocol for CHO cells.

Recombinant DNA constructs and expression plasmids

The plasmid expression vector pPG5 (23), which uses the SV40 early promoter and polyadenylation site for expression of the cloned insert, and G418 resistance for selection of stable transfectants, was used in all transfection experiments, except for tetracycline-inducible expression and for expression of the hamster class I-gfp fusion protein (see below). A cDNA-encoding wild-type H-2K^k (from P. Kourilsky, Institut Pasteur) was subcloned into the BamHI restriction enzyme site of the pBamHI site (into which the CHO cDNA sequence had been cloned). Therefore, creating the Kd expression plasmid with HindIII, removal of a 300-bp fragment, and religation generated a new expression plasmid encoding the leader sequence and the first 286 aa of mature Kd, followed by the additional amino acids ALA and ASP and terminating before the transmembrane domain. A similar construct expressing the first 284 aa of Kd as a functional soluble molecule has been described previously (24). A single chain version of Kd (Kd Fc) with mouse IgG1 Fc and the mature Kd H chain sequence via a flexible linker was generated using a similar approach to that described previously (25, 26). PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA) and cDNA clone templates was used to generate products encoding mouse β2m and the Kd H chain with overlapping sequences encoding a 15-aa peptide linker. The oligodeoxynucleotides used were 5'-TGCTTCTGCAGTCTTGCAAGCATGCCAGTGAGGTATTTC-3' and 5'-CCGCCCGAACCGCCACCTCCCATGTCTCGATCCCAGTAGAC-3' for β2m and 5'-GGGGTCTGGGTTGGCGGCGTCCCTGCGCGCGCTGGCGGCGGCGGTGGTGTCACG-3' for Kd and 5'-TTACGGTACAGAGAGGCTCTTCG-3' for the Kd H chain. The two PCR products were mixed and spliced by overlap extension, to generate a 1.4-kbp DNA fragment encoding full-length β2m linked via the spacer amino acid sequence (GGGGS), to the N terminus of the mature (no hydrophobic signal sequence) Kd H chain. This fragment was cloned into the expression vector pPG5 (described above), and its complete sequence was verified by automated DNA sequencing (Genetics Core Facility, Imperial College). The cDNA clone encoding the HA of influenza virus A/PR/8/34 (Mount Sinai strain) has been described before (23), and was also cloned into the expression vector pPG5. A 100 bp cDNA clone in expression plasmid pPG5 was obtained from A. Townsend (University of Oxford).

For tetracycline-inducible expression, the full-length cDNA encoding Kd H chain was subcloned into the BamHI restriction enzyme site of the plasmid pcDNA5/TO (Invitrogen Life Technologies), which uses the CMV promoter for expression and allows selection of stable transfectants with hygromycin B. For the generation of the CHO class I cDNA fused to GFP, the RT-PCR product described above was subcloned into the plasmid pcDNA3.1/CT-GFP-TOPO, using a GFP fusion TOPO TA expression plasmid (Invitrogen Life Technologies). This generates the full-length CHO class I Kd H chain with its C terminus fused to GFP (cycle 3), separated by a 17-aa linker sequence. The construct was checked by DNA sequencing, and the CHO MHC class I sequence was identical with that reported previously, accession AY064389 (22).

Analysis of MHC class I cell surface expression by flow cytometry

CHO cells were harvested using PBS containing 0.5 mM EDTA, and 5 x 10^6 cells were incubated with primary and, when necessary, secondary Abs on ice for 30 min each. Cells were washed twice in PBS between and after staining, resuspended in 0.5 ml of PBS, and analyzed on an EPICS XL flow cytometer using Expo 32 software (Beckman Coulter).

Confocal microscopy

Immediately after transient transfection, CHO cells were spotted onto glass microscope slides in culture medium, and incubated for 24 h to allow protein expression and cell adherence. The cells were then washed with...
PBS, and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. The cells were washed again three times with PBS, mounted in MOWIOL 4-88 (CN Biosciences), and viewed with a Zeiss LSM5 Pascal microscope using a ×63/1.4 objective. For colocalization experiments, after fixation cells were permeabilized using 1% Triton X-100 in PBS for 10 min, and stained with 10 μg/ml anti-protein disulfide isomerase Ab for 1 h at 37°C, followed by Alexa Fluor 568 goat anti-mouse IgG1 secondary Ab. Multichannel acquisition was used to avoid cross talk between the respective channels.

**Immunoprecipitation and two-dimensional gel electrophoresis**

Cells were metabolically labeled for 1 h with 0.7 MBq Trans35S-label (ICN Biomedicals) and lysed in 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris, pH 7.5, 1 mM PMSF). Precleared, postnuclear lysates were immunoprecipitated with the appropriate mAb or antiserum, followed by protein G-Sepharose. The immunoprecipitates of CHO class I molecules were analyzed by SDS-PAGE on a 15% gel. For immunoprecipitates from SP3 cells, two-dimensional gel electrophoresis was performed, as described previously (27).

**Results**

**MHC class I competition in transfected CHO cells**

During studies on MHC class I-restricted Ag presentation, often it has been convenient to transfet an MHC class I H chain into a cell line that does not normally express that class I allele. For example, this approach has been useful to define which particular class I molecule acts as the restriction element for a specific peptide epitope. Typically, the expression vectors used contain a strong viral promoter, so that the cloned gene of interest is overexpressed. With MHC class I H chain transfections, we have observed that sometimes the introduction of a new class I allele causes down-regulation of one or more of the endogenous class I molecules at the cell surface. However, this is not always the case; transfection of certain class I molecules has little effect on the endogenous class I molecules, even when the transfected molecule is efficiently expressed.

Fig. 1 shows an example of strong competition for cell surface expression between class I molecules in stably transfected cells. When the H-2Kd H chain was expressed from the SV40 early promoter in CHO cells, there was a reproducible reduction of ~10-fold in the cell surface expression of an endogenous hamster classical MHC class I molecule (Fig. 1a), as determined using the mAb B22 (22). Analogous to the findings in F1 hybrid and MHC class I transgenic mice we reported previously (17), there was competition between the class I alleles for cell surface expression. This effect was not an artifact of one particular transfected clone, because double staining of uncloned, polyclonal stable transfec-
tants showed the same effect (Fig. 1c). This approach had the advantage of providing an internal control population of transfected cells not expressing Kd, a result of using nonlinearized plasmid DNA for transfections. The inhibition of expression of endogenous hamster class I molecules was found consistently, even at widely different times after the selection of transfec-
tants. To investigate whether the competition was a specific effect, or could be caused by transfection of CHO cells with any cell surface glycoprotein, the same expression vector and transfection protocol were used to introduce an influenza A virus hemagglutinin (HA) into CHO cells, and the effects on hamster class I expression were measured (Fig. 2). Although it is known that HA expression at the cell surface is subject to rigorous quality control in the ER, and HA is able to associate with the molecular chaperones calnexin and calreti-
culin, which are also implicated in the class I presentation pathway (28), HA expression had no effect on the surface levels of the endogenous hamster class I molecules (Fig. 2). Therefore, the competition seemed to occur specifically between MHC class I molecules.

To rule out the possibility that the competition was an artifact of transfection and selection, two further approaches were used to express Kd in CHO cells (Fig. 3). First, a tetracycline-inducible expression system was used (see Materials and Methods). Double staining of uncloned, polyclonal stable transfec-
tants after culture in media containing tetracycline demonstrated that Kd expression was required for the observed reduction in cell surface expression of hamster class I molecules. Incubation of untransfected CHO cells with tetracycline had no effect on B22 staining (data not shown). Therefore, the competitive effect was not due to transfection with Kd expression plasmid per se. Second, we reasoned that transient transfection of Kd into CHO cells (with no selection of transfec-
tants) should also show the same competitive effect. This was indeed the case, and double staining of the total cell populations 24 and 48 h after transfection again showed that Kd expression caused down-regulation of cell surface CHO class I expression (Fig. 3b). Furthermore, these staining profiles indicated that the effect was true competition, because cells with lower levels of Kd expression showed less down-regulation of CHO class I.
These results showed that the competition was not an artifact of transfection and selection, but it remained possible that the competition occurred at the level of mRNA, rather than the protein. To exclude this possibility, we mutated the ATG start codon of Kd in the expression plasmid used for the experiment shown in Fig. 3b to AGG, so that although mRNA should still be produced in transfected cells, no Kd protein would be made. When this mutant Kd expression plasmid was transiently transfected into CHO cells, there was no significant down-regulation of CHO class I cell surface expression (Fig. 3c), even though the level of Kd-specific mRNA was similar to that of cells transfected with the wild-type Kd plasmid, with at most a 2-fold difference (Fig. 3d). This result strongly suggested that the competition occurs between different class I proteins.

**Kd expression inhibits export from the ER and correct assembly of CHO class I molecules**

To confirm that the competition occurred between class I proteins, and to determine the intracellular location of class I molecules that were prevented from reaching the cell surface, an expression construct was generated to fuse GFP to the C terminus of the CHO class I molecule recognized by the B22 mAb (see Materials and Methods). This construct was transiently transfected into either CHO cells, or into CHO cells stably expressing Kd, and 24 h later the pattern of GFP fluorescence was determined by confocal microscopy (Fig. 4a). As expected, in CHO cells, GFP fluorescence was largely on the cell surface, indicating cell surface expression of the transfected hamster class I molecule. However, a different pattern of GFP fluorescence was observed when the same construct was transfected into CHO cells already expressing Kd (Fig. 4a). In these cells, there was very little cell surface expression; instead, the fluorescence indicated hamster class I molecule expression surrounding the nucleus, with a network of fluorescence extending into the cytosol. This pattern is characteristic of the distribution of the ER. To confirm that this was indeed expression in the ER, two-color staining was performed using an Ab against protein disulfide isomerase, an abundant resident protein of the ER. This showed that the GFP fluorescence colocalized with staining for the ER (Fig. 4b). Therefore, although the GFP-class I fusion protein was synthesized in CHO cells expressing Kd, it was unable to reach the cell surface and was retained within the ER, where class I molecules are known to assemble.

There were two possibilities to explain this effect of Kd expression: either Kd prevents the correct assembly of CHO class I molecules (and therefore their export from the ER), or Kd competes directly with fully assembled CHO class I molecules for transport out of the ER. To distinguish between these two possibilities, the B22 mAb was used to immunoprecipitate hamster class I molecules from lysates of CHO cells or CHO cells expressing Kd.
5). B22 only recognizes correctly folded class I molecules assembled with peptide (29), and this experiment showed that the presence of Kd prevented the correct assembly of CHO class I H chains (Fig. 5). It is possible that the B22 Ab displays some peptide preference in the assembled CHO class I molecules it recognizes, but nevertheless, the competitive effect of Kd H chain expression in CHO cells was very clear.

Competition between MHC class I molecules in CHO cells is not for β2m, or for access to TAP and loading with high affinity peptide

The results described above demonstrated that Kd H chain protein competes with CHO class I protein for folding and assembly in the ER. This competition was most likely to be for the two other components required to form a stable class I molecule complex: β2m and peptide. To test whether β2m was the limiting factor for class I expression in transfected CHO cells, a single chain version of Kd was engineered in which mouse β2m was covalently fused to the N terminus of the mature Kd H chain via a flexible linker sequence (see Materials and Methods). This molecule should not be able to associate with endogenous β2m, and therefore, should not compete with endogenous hamster class I molecules for β2m, if this was the mechanism of the competition. As shown in Fig. 6a, the single chain Kd construct did cause down-regulation of cell surface hamster class I molecules in CHO cells, although to a lesser extent than wild-type Kd. The Kd H chain in these cells was associated with

FIGURE 4. Localization of a hamster class I H chain-GFP fusion transiently transfected into CHO cells. See Materials and Methods for details. a, The GFP fusion was transfected into either CHO cells or CHO cells expressing Kd, as indicated. Two examples of each GFP expression pattern are shown. b, The GFP fusion was transfected into CHO cells expressing Kd, and cells were stained for the ER marker protein disulfide isomerase (red) or viewed for GFP fluorescence (green). Bottom panel, Shows an overlay of both channels, with colocalization in yellow.

FIGURE 5. Immunoprecipitation of folded hamster class I molecules from CHO cells or from CHO cells expressing Kd. Cells were metabolically labeled and lysed, as described in Materials and Methods. B22-249 immunoprecipitates were analyzed by SDS-PAGE and autoradiography. The positions of class I H chain and β2m are indicated.

FIGURE 6. Competition between class I H chains in CHO cells is not for β2m. CHO cells were stably transfected with either the Kd H chain alone or a with a single chain Kd-mouse β2m fusion construct, and polyclonal (uncloned) populations were double stained for Kd expression and hamster class I expression (a), or stained with M1/42 for detection of Kd H chains associated with mouse β2m (b).
mouse β₂m, as expected, because the cells were positive for staining with M1/42 (Fig. 6b), a mAb that recognizes mouse class I H chains only when they are associated with mouse β₂m (30, 31). Therefore, we conclude that the competition between class I molecules in transfected CHO cells is not for binding to β₂m.

The other most likely possibility was competition for loading of class I with peptide. To test this possibility, we generated a mutant form of the K<sup>d</sup> H chain. Several groups have described the effects of a nonconservative point mutation at position 134 of the class I H chain that changes threonine to lysine (T134K) (32–34). This mutation abolished the ability of the class I H chain to bind to tapasin, TAP, and calreticulin, and as a result the class I molecules were transported rapidly to the cell surface in a relatively unstable form, loaded with suboptimal peptides. This point mutation was introduced into the K<sup>d</sup> H chain, and the construct was transfected into CHO cells. Double staining of uncloned, polyclonal stable transfectants (Fig. 7) showed that, although cell surface expression was reduced to 30% of that found with normal K<sup>d</sup> (as expected for the T134K phenotype), the level of down-regulation of surface hamster class I expression was the same as that found with wild-type K<sup>d</sup> H chain. Surprisingly, interaction between the class I H chain and the peptide-loading complex was not important for the competition to occur. This result strongly suggested that the competition between class I H chains was occurring at an early stage of the assembly pathway before association with TAP, and was not for high affinity peptide. We also generated a construct that expressed a soluble version of K<sup>d</sup> that was not anchored in the cell membrane (see Materials and Methods). Soluble K<sup>d</sup> was also equally efficient at down-regulating cell surface expression of hamster class I molecules (Fig. 7), even though it was not present in the plasma membrane.

**Competition between MHC class I H chains occurs in TAP2-negative CHO cells**

The results described above indicated that the mechanism of class I competition did not involve TAP and the peptide-loading complex. Therefore, we reasoned that such competition should also occur in TAP-negative cells. Under normal conditions, TAP-negative cells only express very low levels of assembled class I molecules at their cell surface because of the lack of supply of appropriate peptides, and so flow cytometry would not be able to detect any class I competition. However, at reduced incubation temperatures, the normally unstable class I molecules are readily detectable at the surface of TAP-deficient cells (35). TAP2-negative CHO cells have been described (20), and we confirmed that B22 staining of these cells cultured at 37°C was very low, and that there was a substantial increase in staining when cells were incubated at 26°C overnight (data not shown). Therefore, K<sup>d</sup> H chain was transfected into TAP2-negative CHO cells, and uncloned, polyclonal stable transfectants incubated overnight at the two different temperatures were double stained and analyzed by flow cytometry (Fig. 8). The results showed that there was a substantial increase in B22 staining of K<sup>d</sup>-negative cells at the lower temperature, but that there was no such increase in the K<sup>d</sup>-positive cell population (Fig. 8). Therefore, the competition was still occurring, and the K<sup>d</sup> H chain was able to down-regulate CHO class I molecules even in TAP-negative cells. There was a substantial increase in K<sup>d</sup> cell surface expression in TAP2-negative CHO cells incubated at 26°C; these cells may be useful in peptide-binding assays for the characterization of K<sup>d</sup>-restricted epitopes.

**Competition occurs between different mouse class I H chains in transfected mouse cells**

Our previous work using F<sub>1</sub> hybrid and MHC class I transgenic mice demonstrated that competition also occurs between different
mouse class I H chains in mouse cells (17), showing that the effect is not just a result of cross-species transfections. We have also observed competition between different mouse class I H chains in transfected mouse cells, but the level of competition is significantly lower than that described above for the effects of K\(^d\) expression in CHO cells. Fig. 9 shows the effects of transfecting the D\(^b\) class I H chain into the H-2\(^k\) cell line SP3 (19). D\(^b\) expression caused a 2.5- to 3-fold decrease in cell surface expression of the endogenous K\(^k\) class I molecule. To investigate the mechanism of this effect, we took advantage of an anti-exon 8 antiserum that recognizes the cytoplasmic tail of K\(^k\) molecules, but does not recognize the other class I molecules present in the transfected SP3 cells, namely D\(^k\) and D\(^b\). This antiserum recognizes K\(^k\) class I H chains independently of their folding and assembly status, allowing detection of all K\(^k\) H chains in cells. Immunoprecipitates with this antiserum of lysates from SP3 and SP3-D\(^b\) cells were analyzed by two-dimensional electrophoresis (see Materials and Methods) (Fig. 10b). The results showed that although both cell lines expressed similar levels of K\(^k\) H chains, the amount of β\(^m_2\)m associated with the K\(^k\) H chain was significantly reduced in the cells expressing D\(^b\). Therefore, the presence of D\(^b\) inhibited the association of K\(^k\) H chain with β\(^m_2\)m, consistent with competition between the different mouse class I H chains at an early stage of the assembly pathway, and also consistent with the results obtained in transfected CHO cells. SP3 and SP3-D\(^b\) cell lysates were also immunoprecipitated with anti-calreticulin antiserum, and analyzed in the same way (Fig. 10c). The presence of D\(^b\) caused a decrease in the amount of K\(^k\) H chain associated with calreticulin, a component of the peptide-loading complex, again consistent with competition at an early stage of the class I assembly pathway.

**Discussion**

In this study, we show that different MHC class I H chains can compete with each other at an early stage of their intracellular assembly, and that this may lead to large alterations in cell surface expression levels of assembled class I molecules. Although it is now widely accepted that there is competition between T cells for MHC molecules that present Ag on APCs (36), the concept of competition between MHC molecules within a cell has received little attention. However, such intracellular competition may have a significant effect in shaping T cell responses. We showed that the competition between class I H chains is not competition for the L chain β\(^m_2\)m, nor for association with TAP and the peptide-loading complex, and therefore, not for high affinity peptide. In addition, the competition also occurred in cells lacking functional TAP, and prevented class I H chains from associating with β\(^m_2\)m. Taken together, these results show that the competition must occur at an early stage of class I H chain folding.

Newly synthesized class I H chain/β\(^m_2\)m heterodimers are found in the ER bound to at least four other proteins that are thought to be involved in their assembly with peptides: TAP, tapasin, ERp57, and calreticulin, which together constitute the peptide-loading complex (37). Before their incorporation into the peptide-loading complex, unfolded MHC class I H chains associate with the chaperone calnexin (38, 39), and possibly with the oxidoreductase ERp57, although this has been controversial (40–42). Competition between class I H chains for folding at a very early stage is likely to be for access to one of these proteins, unless there are other, as yet unknown components of the folding pathway. Although calnexin clearly can facilitate MHC class I H chain folding (43), class I assembly was apparently unimpaired in a calnexin-negative cell line (44, 45), suggesting that competition was not for association with calnexin. It has been shown that a T134K mutant class I H chain was unable to associate with ERp57 (42, 46), so that if MHC molecules competed for access to ERp57, this type of mutant should not be able to compete with other class I H chains. However, as shown in Fig. 7, we found that T134K-K\(^{d*}\) was as effective a competitor as wild-type K\(^{d*}\), strongly suggesting that the competition was not for ERp57. Therefore, further work will be required to define precisely the mechanism of competition between class I H chains. We favor the hypothesis that the competition occurs at

![FIGURE 9. Competition occurs between mouse class I H chains in mouse cells. SP3 cells (H-2\(^k\) haplotype) and SP3 cells stably transfected with D\(^b\) were stained for either K\(^k\) (endogenous class I) or for D\(^b\) (transgene), and analyzed by flow cytometry.](Image 323x521 to 539x742)

![FIGURE 10. Analysis of K\(^k\) H chain expression and chaperone interaction in SP3 cells and SP3 cells expressing D\(^b\). Cells were metabolically labeled and lysed, as described in Materials and Methods, and lysates were immunoprecipitated with: a, no Ab; b, anti-exon 8 antiserum (to detect all K\(^k\) H chains irrespective of their conformation); or c, anti-calreticulin antiserum. Immunoprecipitates were analyzed by two-dimensional gel electrophoresis and autoradiography. The positions of K\(^k\) H chain, β\(^m_2\)m, D\(^b\) H chain, and calreticulin (CRT) are indicated.](Image 119x540 to 227x742)
the initial stage of H chain folding into a form competent to associate with \(\beta_2\)-m, because in mouse cells demonstrating competition we have been unable to rescue correctly folded class I molecules by the addition of excess high affinity peptide and \(\beta_2\)-m to cell lysates (data not shown). This argues against other possibilities, such as competition for binding peptides of low or moderate affinity. Future experiments will follow the fate of tagged class I molecules subject to strong competition, and investigate their association with components of the ER.

There is some published evidence that MHC class I molecules can compete under normal physiological circumstances. When the \(K^\alpha\) gene was removed from H-2\(^b\) mice by targeted deletion, there was a \(\sim 50\%\) increase in cell surface expression of the remaining classical class I molecule \(D^\alpha\), measured on T lymphocytes (47). The potential for competition certainly exists, especially in human cells, which express up to six different classical HLA class I molecules. It has been reported that it is the level of class I H chain, not \(\beta_2\)-m or peptide, that controls the level of cell surface expression of HLA class I molecules under normal circumstances (48).

The concept of competition between different MHC class I molecules affecting their function has not generally been considered, but our results show that it can take place, and because of its potential importance in shaping T cell responses and determining susceptibility to disease, we propose that it deserves further investigation.

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References

reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. Proc. Natl. Acad. Sci. USA 89:4734.


