Evidence for successive peptide binding and quality control stages during MHC class I assembly

Jonathan W. Lewis and Tim Elliott

Intracellular antigens are continually presented to cytotoxic T lymphocytes by major histocompatibility complex (MHC) class I molecules, which consist of a polymorphic 43 kDa heavy chain and a 12 kDa soluble subunit β2-microglobulin (β2m), and which bind an 8–10 amino-acid antigenic peptide. The assembly of this trimolecular complex takes place in the lumen of the endoplasmic reticulum (ER) [1] and almost certainly requires cofactors. Most MHC class I molecules in the ER that have not yet acquired peptide are simultaneously bound to the transporter associated with antigen processing (TAP), to the 48 kDa glycoprotein tapasin and to the lectin-like chaperone calreticulin, in a multicomponent 'loading complex' [2]. Previous studies have shown that a mutant MHC class I molecule T134K (in which Thr134 was changed to Lys) fails to bind to TAP [3]. Here, we show that this point mutation also disrupted, directly or indirectly, the interaction between MHC class I molecules and calreticulin. T134K molecules did not present viral antigens to T cells even though they bound peptide and β2m normally *in vitro*. They exited the ER rapidly as 'empty' MHC class I complexes, unlike empty wild-type molecules which are retained in the ER and degraded. We show here that, paradoxically, the rapid exit of empty T134K molecules from the ER was dependent on a TAP-derived supply of peptides. This implies that MHC class I assembly is a two-stage process: initial binding of suboptimal peptides is followed by peptide optimisation that depends on temporary ER retention.

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Received: 10 February 1998
Revised: 26 March 1998
Accepted: 17 April 1998

Published: 25 May 1998

Current Biology 1998, 8:717–720
http://biomednet.com/elecref/0960982200800717

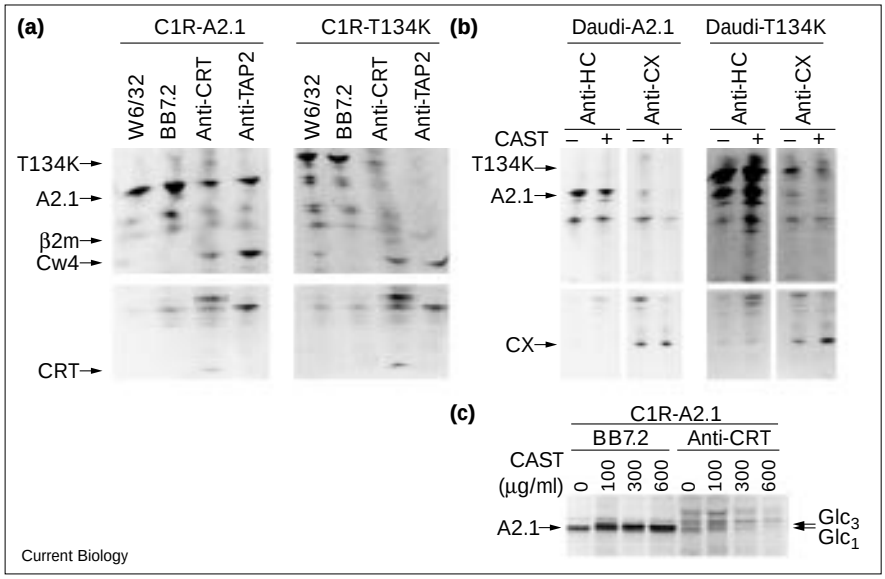
 ISSN 0960-9822

Results and discussion

In addition to the failure of T134K molecules to interact with the TAP1–TAP2 heterodimer, Figure 1a shows that they did not associate with the ER-resident protein calreticulin either. Both human leukocyte antigens (HLAs) HLA-A*0201 and HLA-Cw4 could be recovered by coimmunoprecipitation with both anti-TAP2 and anti-calreticulin antibodies in digitonin lysates, but no association with T134K was detected. T134K did, however, interact with the ER chaperone calnexin — a protein that is homologous to calreticulin — in the β2m-negative cell line Daudi (Figure 1b). These experiments demonstrate that whereas calreticulin can discriminate between the wild-type HLA-A*0201 and the T134K mutant, calnexin cannot. The basis for this discrimination is unclear: both calreticulin and calnexin are reported to have very similar, if not identical, substrate specificities and have been shown to bind preferentially to monoglucosylated trimming intermediates of glycoproteins in the ER ([4,5], reviewed in [6,7]). Consistent with this, the interaction between calnexin and HLA-A*0201 or T134K is sensitive to castanospermine, a compound that inhibits the trimming of the immature glycan on the heavy chain by glucosidase I and II (Figure 1b). Figure 1c shows that castanospermine also inhibits the interaction between HLA-A*0201 and calreticulin in a dose-dependent way. Given that Thr134 is at a position distal to the single glycosylation site (Asn86) of human MHC class I molecules, as seen in the three-dimensional structure [8], these results suggest that, in addition to the glycan-dependent aspect of calreticulin binding to MHC class I molecules, other aspects contribute to this interaction.

The failure of T134K to interact with calreticulin could be due to the direct disruption of the calreticulin-binding site on the class I heavy chain, or could be an indirect effect via the disruption of an interaction with TAP, tapasin or some other cofactor. It is possible, for example, that the interaction between MHC class I and calreticulin is dependent on a castanospermine-sensitive interaction with another cofactor such as tapasin. Figure 2 shows that the binding of calreticulin, tapasin and TAP to MHC class I was cooperative. Thus, the amount of HLA-A*0201 recovered from cells with anti-calreticulin antiserum was drastically reduced when either TAP (in T2 cells) or tapasin (in .220 cells) were missing (Figure 2b). To date, it has not been possible to look at the association between TAP, tapasin and HLA-A*0201 in cells that lack calreticulin. It is possible, however, to block the calreticulin–MHC class I interaction with castanospermine. Figure 2c shows that the interaction between HLA-A*0201 and TAP was sensitive to the inhibition of glycan trimming by castanospermine. Like the calreticulin–MHC class I interaction, there was a dose-dependent effect of castanospermine on the TAP–MHC class I interaction (Figure 2d). These experiments indicate

Figure 1



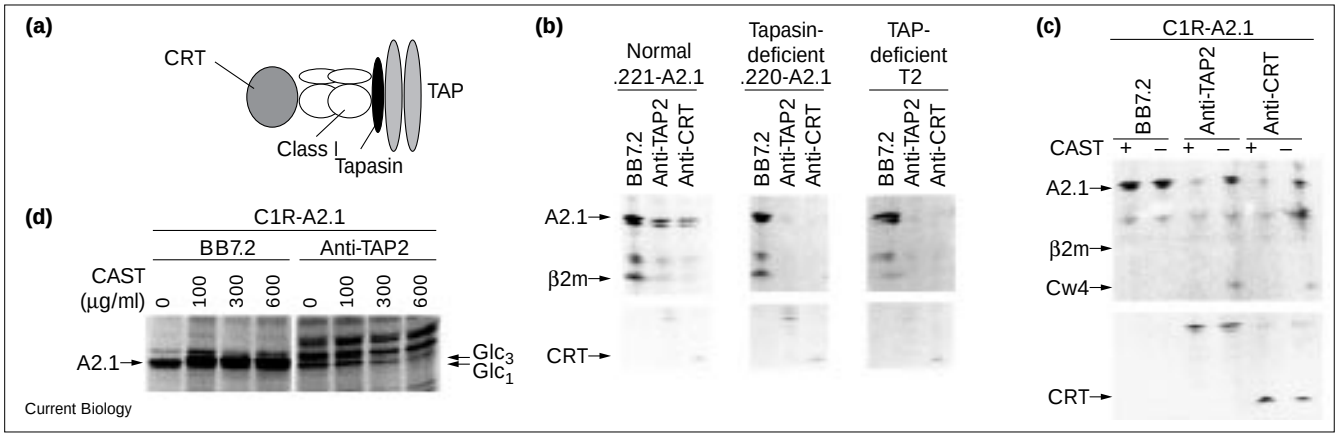
T134K interacts with calnexin (CX) but does not associate with calreticulin (CRT). (a) Metabolically labelled lysates of C1R cells transfected with HLA-A*0201 (C1R-A2.1) or T134K (C1R-T134K) were immunoprecipitated with monoclonal antibodies W6/32 (anti-HLA-A,B,C) and BB7.2 (anti-HLA-A2) or with anti-calreticulin or anti-TAP2 antisera. The immunoprecipitates were analysed by one-dimensional isoelectric focusing (1D-IEF) to resolve coprecipitating HLA-A*0201 (A2.1) and HLA-Cw4. T134K migrated higher than wild-type HLA-A*0201 in the 1D-IEF gel, due to the addition of positive charge by the threonine-to-lysine mutation. HLA-Cw4 was co-precipitated equally well from lysates of either C1R-A2.1 or C1R-T134K cells, and served as an internal control. (b) β2m-deficient Daudi cells were infected with recombinant vaccinia virus expressing either HLA-A*0201 or T134K. (Daudi cells were used because calnexin has been shown to bind to free class I heavy chain in the absence of β2m; the association of HLA-A*0201 and T134K with calnexin is therefore shown best in β2m-deficient cells.) Infected cells were then split in half; half were not treated and half were treated with 600 μg/ml castanospermine (CAST) at a concentration known to prevent trimming of precursor triglucosylated N-linked glycans Glc_3 to the monoglucosylated Glc_1 form (this trimming is required for calnexin to bind to class I heavy chains). Metabolically labelled lysates were then immunoprecipitated with either anti-heavy chain (anti-HC) or anti-calnexin (anti-CX) antisera. The top and bottom of the 1D-IEF gels are shown to save space. (c) The interaction between HLA-A*0201 and calreticulin is carbohydrate dependent. C1R-A2.1 cells were treated with an increasing concentration of castanospermine (0, 100, 300, 600 μg/ml). Metabolically labelled HLA-A*0201 molecules were then immunoprecipitated with BB7.2 antibody or coimmunoprecipitated with anti-calreticulin antiserum. The effectiveness of castanospermine was indicated by the change in mobility of HLA-A*0201 in the SDS polyacrylamide gel as the dose of castanospermine increased, corresponding to the inhibition of glucose trimming from the triglucosyl Glc_3 to monoglucosyl Glc_1 core glycan. Note that only the fast mobility Glc_1 is associated with calreticulin. The band migrating just above the Glc_3 heavy chain band in the anti-calreticulin precipitations is most probably tapasin (data not shown).

that the failure of T134K to interact with calreticulin could be secondary to its failure to interact with other cofactors in the loading complex. They also show that a stable interaction between class I molecules and each member of this complex is dependent on the presence of the other component members.

T134K molecules are released into the secretory pathway prematurely as peptide-receptive molecules [3]. They escape degradation in the ER — which is the usual fate of peptide-receptive class I molecules synthesised in cells in which the supply of peptides to the ER is limited. If T134K was bypassing the 'peptide loading' step of antigen presentation, we would expect this rapid transport of empty mutant molecules to be insensitive to the presence of a functional TAP.

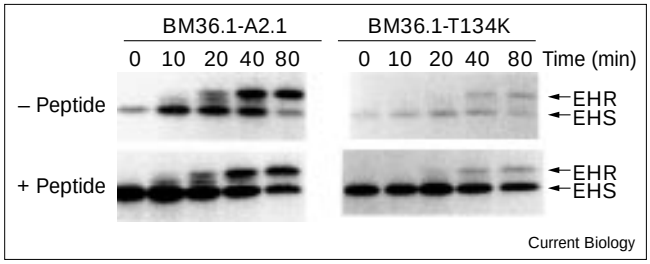
To test this, we introduced T134K into cell lines that are unable to supply newly synthesised class I molecules with TAP-derived peptides because of an absent or defective TAP complex. In this case, only a TAP-independent peptide pool would exist in the ER. In normal C1R cells, around 70% of wild-type HLA-A*0201 leaves the ER stably bound to peptides, whereas the remaining 30% of molecules are not stabilised, and as a result remain in the ER to be degraded [3]. In contrast, only a few (14%) T134K molecules leave the ER stably bound to peptides; the majority (70%) exit in a peptide-receptive state, with very few molecules being retained in the ER [3]. The intracellular fate of both T134K and normal HLA-A*0201 expressed in the TAP-defective cell line BM36.1 [9] was then assessed for comparison. Figure 3 shows that in BM36.1 cells, the majority of T134K molecules, like HLA-A*0201, did not exit the ER but were retained as unstable complexes that were sensitive to endoglycosidase H (87% and 61% for T134K and HLA-A*0201, respectively). Only about 10% of T134K molecules and 30% of wild-type HLA-A*0201 molecules exited the ER in BM36.1 cells as stable complexes. Thus, we show for the first time that although a TAP-derived supply of peptides to the ER is necessary to ensure the stable peptide-dependent assembly of MHC class I molecules and the presentation of endogenous antigens to cytotoxic T lymphocytes, it is not sufficient. A supply of TAP-derived peptides is

Figure 2



The binding of calreticulin (CRT), tapasin and TAP to HLA-A*0201 (A2.1) is cooperative. **(a)** A schematic representation of the multicomponent loading complex. Metabolically labelled lysates of **(b)** normal .221-A2.1 cells, tapasin-deficient .220-A2.1 cells and TAP-deficient T2 cells and of **(c)** C1R-A2.1 cells (mock-treated or treated with 600 μg/ml castanospermine, CAST) were immunoprecipitated with BB7.2 (anti-HLA-A2) antibody or with anti-TAP2 or anti-calreticulin antisera. The immunoprecipitates were analysed by 1D-IEF to resolve coprecipitating HLA-A*0201 and HLA-Cw4. Note that less HLA-A*0201 associated with calreticulin when cells lacked either tapasin (.220-A2.1) or TAP (T2). When the interaction between calreticulin and HLA-A*0201 was disrupted with castanospermine (c), less HLA-A*0201 coprecipitated with TAP. **(d)** Metabolically labelled cell lysates of C1R-A2.1 cells treated with castanospermine at the indicated concentrations were immunoprecipitated with BB7.2 (anti-HLA-A2) antibody or with anti-TAP2 antiserum. Immunoprecipitates were analysed by 10% SDS–PAGE. As the dose of castanospermine increased, the amount of HLA-A*0201 associated with TAP decreased. TAP associated only with the Glc_1 form of HLA-A*0201.

Figure 3



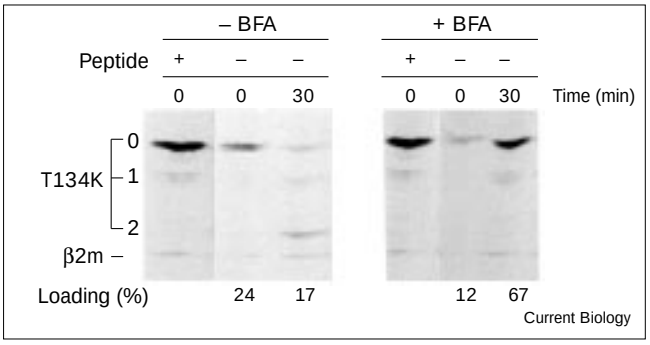
BM36.1 cells were infected with recombinant vaccinia virus expressing either HLA-A*0201 (A2.1) or T134K. Cells were metabolically labelled and chased for the time periods indicated. BB7.2-reactive class I complexes were then recovered from cell lysates after overnight pre-clearing in the absence (– peptide) or presence (+ peptide) of a saturating concentration (20 μM) of stabilising HIV pol peptide. Only heavy-chain bands are shown because human β2m is poorly visualised following a 10 min pulse-label. Intracellular maturation was determined by the sensitivity of class I molecules to endoglycosidase H (EHS indicates endoglycosidase-H-sensitive; EHR indicates endoglycosidase-H-resistant). The intracellular fate of class I complexes in each cell line could be quantified (see Supplementary material): from each chase, the proportion of stable class I complexes that exited the ER was 32% for HLA-A*0201 and 11% for T134K; the proportion of 'peptide-receptive', unstable complexes that exited was 7% for HLA-A*0201 and 2% for T134K; the proportion of class I molecules that remained in the ER was 61% for HLA-A*0201 and 87% for T134K. When C1R cells were infected with the recombinant vaccinia viruses, both T134K and wild-type HLA-A*0201 exited the ER and became endoglycosidase-H-resistant; this is also the case when T134K and HLA-A*0201 are expressed from transfected genes [3].

clearly essential to allow T134K molecules to escape degradation and to exit the ER. They nevertheless enter the secretory pathway in a peptide-receptive state when assayed *in vitro*. This suggests that the mutation acts to prevent class I molecules from optimising the binding of their peptide ligand, rather than preventing peptide binding per se. It is this failure to optimise the binding of the peptide ligand that leads to the egress of unstable class I molecules from the ER.

The optimisation of peptide loading onto class I complexes may be linked to their retention time in the ER. We have investigated this proposal using the drug brefeldin A (BFA), which promotes the retention of newly synthesised glycoproteins in the ER by reorganising the structure of the ER and Golgi apparatus. Figure 4 shows that when C1R-T134K cells were incubated with BFA for 30 minutes (the approximate half-time for egress of wild-type HLA-A*0201 molecules from the ER, compared with 12 minutes for T134K), the fraction of stable molecules increased dramatically from 17% in the absence of BFA to 67% in its presence. This indicates that ER retention alone may facilitate the assembly of class I molecules with optimal peptides.

Mechanisms by which peptide optimisation could be achieved include peptide exchange, or the enzymatic trimming of suboptimal peptides to a suitable length for class I binding [10,11]. In support of the peptide exchange mechanism, Sijts and Pamer [12] have shown recently that

Figure 4



Retention in the ER promotes intracellular peptide loading of T134K. Cells metabolically labelled for 5 min were incubated in the absence or presence of 10 μM BFA before they were lysed. The number of sialic acid residues present on the T134K molecules is labeled. T134K that became stably assembled was calculated as a fraction of the total cohort (visualised at t = 0 by the addition of stabilising peptide). Note that T134K becomes di-sialylated only in the absence of BFA.

peptide dissociation from class I molecules in the ER is enhanced compared with the cell surface and yet ER-resident class I molecules have a greater capacity to bind new epitopes. This process might be a 'facilitated exchange' process, requiring the assistance of a separate cofactor similar to the role of HLA-DM described for the class II pathway (reviewed in [13]). As for MHC class II, there might be varying degrees of dependency on such a cofactor between alleles in order to bring about effective peptide exchange [14–16].

Materials and methods

Cell lines, antibodies and peptides

The TAP-deficient .174 cells, tapasin-deficient .220 cells, and tapasin-competent .221 cells, as well as .220-A2.1 and .221-A2.1 cells, were the generous gifts of R. DeMars. BM28.7 [9] and TAP-defective BM36.1 cells were a gift from A. Ziegler. T2 cells were a gift from P. Cresswell. C1R cells transfected with HLA-A*0201 (C1R-A2.1) and HLA-A*0201T134K (C1R-T134K) were a gift from J. Frelinger. Monoclonal antibodies used were anti-HLA-A2 (BB7.2 [17]) and anti-HLA-A, anti-HLA-B and anti-HLA-C (W6/32 [18]). Rabbit anti-free-heavy-chain and rabbit anti-TAP2 sera were generated by and were a gift from J.J. Neefjes [3]. Rabbit anti-calreticulin was from Affinity Bioreagents. The rabbit antiserum to calnexin was generated by B. Gao to a synthetic peptide corresponding to the 15 carboxy-terminal residues of human calnexin. The HLA-A*0201 binding peptides ILKEPVHGV (HIV pol residues 476–484) and GILGFVFTL (influenza A MP58–66) were synthesised by Research Genetics.

Pulse-chase analysis, immunoprecipitation, coimmunoprecipitation and band analysis

These were performed exactly as described [3]. The fractions of a pulse-labelled cohort of class I molecules which either leave the ER in stable association with peptide, or leave the ER as unstable peptide-receptive complexes, or are retained and degraded in the ER as peptide-receptive complexes were calculated from the integrated optical density (IOD) of endoglycosidase-H-sensitive and endoglycosidase-H-resistant class I heavy chain bands. For details see the Supplementary material.

Viruses

Recombinant vaccinia viruses were made by homologous recombination into the thymidine kinase gene of WR vaccinia as described [11]. For immunoprecipitation experiments, cells were pelleted and infected with recombinant vaccinia at multiplicity of infection (MOI) = 10 for 90 min at 37°C. Cells were then washed once and resuspended in warm medium for 3.5 h prior to metabolic radiolabelling.

Supplementary material

Details of the quantitation of pulse-chase immunoprecipitation are published with this paper on the internet.

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Supplementary material

Evidence for successive peptide binding and quality control stages during MHC class I assembly

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Current Biology 25 May 1998, 8:717–720

The quantitation of pulse-chase immunoprecipitation

Gels were fixed, stained (for SDS polyacrylamide gels), treated with Amplify (Amersham), dried and visualised by autoradiography at –80°C. Bands were quantitated using a MilliGen Bioimage Analyser which expresses band intensities as an integrated optical density (IOD). The fractions of a pulse-labelled cohort of class I molecules which leave the ER in stable association with peptide (A), leave the ER as unstable peptide-receptive complexes (B) or are retained and degraded in the ER as peptide-receptive complexes (C) were calculated from the IOD of endoglycosidase-H-sensitive (EHS) and endoglycosidase-H-resistant (EHR) class I heavy chain bands precipitated by conformation-specific antibodies in the presence or absence of peptide.

A = (maximum IOD EHR HC without peptide)/T (× 100%);

B = [(maximum IOD EHR HC with peptide) – (maximum IOD EHR HC without peptide)]/T (× 100%);

C = [(maximum IOD EHS HC with peptide) – (maximum IOD EHR HC with peptide)]/T (× 100%);

where T = maximum IOD (EHS HC + EHR HC) with peptide. All experiments were performed at least twice and the reproducibility was found to be good. Fractions A, B and C varied less than ±20% from day to day and were not affected by minor differences in the total amount of class I heavy chain synthesised following vaccinia delivery of the heavy chain genes. The accuracy of the calculation was checked by ensuring that the fraction C calculated above equalled the difference between fractions A and B.