Point mutations in the α2 domain of HLA-A2.1 define a functionally relevant interaction with TAP

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Background: Glycoproteins encoded by the major histocompatibility complex class I region (MHC class I) present peptide antigens to cytotoxic T cells (CTLs). Peptides are delivered to the site of MHC class I assembly by the transporter associated with antigen processing (TAP), and cell lines that lack this transporter are unable to present endogenous antigens to CTLs. Although it has been shown that a fraction of newly synthesized class I molecules are in physical association with TAP, it is not known whether this interaction is functionally relevant, or where on the class I molecule the TAP binding site might be.

Results: C1R cells transfected with a mutant HLA-A2.1 heavy chain (HC), where threonine at position 134 in the α2 domain is changed to lysine (T134K), are unable to present endogenous antigens to CTLs. We have studied the biochemistry of this mutant in C1R cells, and found that a large pool of unstable empty class I HC–β_2m (β-2 microglobulin) heterodimers exist that are rapidly transported to the cell surface. The T134K mutant seemed to bind peptide antigens and assemble with β_2m as efficiently as wild-type HLA-A2.1. However, we show here that the inefficiency with which T134K presents intracellular antigen is associated with its inability to interact with the TAP heterodimer.

Conclusions: These experiments establish that the class I–TAP interaction is obligatory for the presentation of peptide epitopes delivered to the endoplasmic reticulum (ER) by TAP. Wild-type HLA-A2.1 molecules in TAP-deficient cells are retained in the ER, whereas T134K is rapidly released to the cell surface, but is unstable, suggesting a role for the TAP complex as an intracellular checkpoint that only affects the release of class I molecules with stably bound peptide ligands.

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Background

Intracellular antigens are presented to cytotoxic T lymphocytes (CTLs) by MHC class I molecules. Class I molecules consist of heavy chain (HC), β-2 microglobulin (β_2m), and antigenic peptides, forming a trimolecular complex. This is transported from the endoplasmic reticulum (ER) to the cell surface where it can be recognized by the T-cell receptor. Correct assembly of the class I–peptide complex in the lumen of the ER is required for stable expression at the plasma membrane. Peptide epitopes seem to come predominantly from proteins degraded in the cytosol (see, for example, [1,2]; reviewed in [3]). These peptides are subsequently translocated across the ER membrane by the heterodimeric transporter associated with antigen processing (TAP; [4–6]). Longer peptides may be trimmed subsequently in the ER to yield epitopes of an optimal size for binding to class I molecules [7–9], and peptides that fail to associate with class I molecules are removed from the ER in an ATP-dependent way [7]. *In vitro* experiments have shown that, as the binding of β_2m and peptide to HC are co-operative, peptide binding and class I assembly are linked phenomena [10]. It is still unclear how the trimolecular complex is assembled *in vivo*, but recent data suggest that assembly of the peptide-receptive HC–β_2m heterodimer occurs in a complex with the ER-resident chaperone protein calnexin [11,12], or the related protein calreticulin (P. Cresswell, personal communication). In addition, a physical association between HC–β_2m heterodimers and the TAP complex has been observed [13,14], and it has been suggested that this interaction is necessary to promote the intracellular loading of peptides onto the newly assembled class I molecules, although peptide loading of class I molecules can also occur in TAP-deficient cells if peptides are delivered directly to the ER in a signal sequence-dependent way [8,15].

In the absence of TAP, HC and β_2m assemble but form an unstable complex as a direct result of their failure to bind peptides [10]. Thus, mutants with defective or absent TAP genes are unable to present epitopes derived from an intracellular source unless they are delivered directly to the ER in a signal-dependent way. Such mutants can, however, present peptides when they are added exogenously in the culture medium, as a proportion of these unstable HC–β_2m molecules reach the cell surface and are peptide-receptive [16,17].

Recently, several C1R transfectant cell lines have been described that express the HLA-A2.1 HC with a variety of single-site point mutations [18–20]. Some of these transfectants, including T134K (where threonine at position 134 is changed to lysine), are unable to present antigen following infection with virus (endogenous antigen), but will effectively present synthetic peptide epitopes when these are added to the culture medium (exogenous antigen) [20]. Here we show not only that C1R-T134K cells have an antigen-presentation phenotype similar to the TAP-negative mutants, but also that, like wild-type HLA-A2.1 expressed in TAP-negative mutants, T134K fails to form stable intracellular complexes with endogenous peptides even though C1R cells express a functional TAP1–TAP2 heterodimer. We show this phenotype can be explained by a defective interaction between T134K and the TAP heterodimer.

Results

T134K expressed in C1R cells produces a .174-like phenotype

It has previously been reported that C1R-T134K cells do not present endogenously synthesized influenza A matrix protein following viral infection, but will present exogenously added peptide epitope [20]. We have shown that the same is true of the HIV-pol(476–484) epitope following infection of C1R-T134K cells with a recombinant vaccinia virus expressing HIV-pol (Table 1). Superficially, this phenotype resembles that of wild-type HLA-A2.1 molecules when they are expressed in the TAP1–TAP2-deficient cell lines .174 and T2 [21–23]. These lines synthesize high levels of MHC class I HC and β_2m, but express low levels of the assembled class I HC–β_2m heterodimers at the cell surface [21]. Intracellular class I molecules are unstable, but can be detected by immunoprecipitation after they have been stabilized by the addition of peptide [17] or β_2m. Each of these observations can be explained by the fact that these mutants lack the ability to supply newly synthesized class I molecules with peptides generated in the cytosol.

To test whether T134K expressed in the TAP1–TAP2-competent cell line C1R displayed any of the other characteristics shown by TAP1–TAP2-negative cell lines, we first determined its cell-surface expression using a fluorescence-activated cell sorter (FACS). In contrast to an earlier report [20], where cell-surface expression of T134K seemed to be normal, we found that C1R-T134K cells expressed A2.1 at the cell surface at around 20 % of wild-type levels, as detected by either BB7.2 or MA2.1 — antibodies specific for determinants on the α1 and α2 domains of native A2.1 molecules (Table 2). Although cell-surface levels are low, this does not reflect low levels of HLA-A2.1 biosynthesis, as equivalent amounts of A2.1 could be immunoprecipitated from lysates of C1R-T134K and C1R-A2.1 cells containing the stabilizing peptide HIV-pol(476–484) (Fig. 1, compare lanes c and e). Interestingly, in the absence of stabilizing peptide, only around 20 % of wild-type levels of T134K could be recovered from lysates by immunoprecipitation with the conformation-sensitive antibody BB7.2 (Fig. 1, compare lanes d and f). These results suggest that T134K expressed in C1R cells behaves in a similar way to wild-type molecules expressed in TAP1–TAP2-negative cell lines (Fig. 1, lanes a and b).

T134K molecules are rapidly transported to the cell surface as unstable complexes

Intracellular maturation of the mutant HLA-A2.1 molecules in C1R cells was compared by pulse–chase analysis with the maturation of wild-type molecules expressed in both C1R and .174 cells. The rate at which A2.1 molecules acquired resistance to endoglycosidase-H (endo-H) was used as a measure of egress from the ER–*cis*-Golgi network to the medial Golgi. At time points during the chase, duplicate samples were taken and lysed in the presence or absence of the stabilizing peptide HIV-pol(476–484). Labelled HLA-A2.1 molecules were then immunoprecipitated with the conformation-sensitive antibody BB7.2.

Table 1

The presentation of HIV-pol(476–484) by C1R-T134K cells following infection by recombinant vaccinia virus.

	C1R-A2.1	C1R-T134K
10 nM peptide*		
10:1†	52‡	67
2:1	40	51
WR vacc		
10:1	7	8
2:1	5	3
HIV-pol vacc		
10:1	30	7
2:1	7	5

*HIV-pol(476–484) (ILKEPVHGV); †effector to target ratio; ‡percent specific lysis.

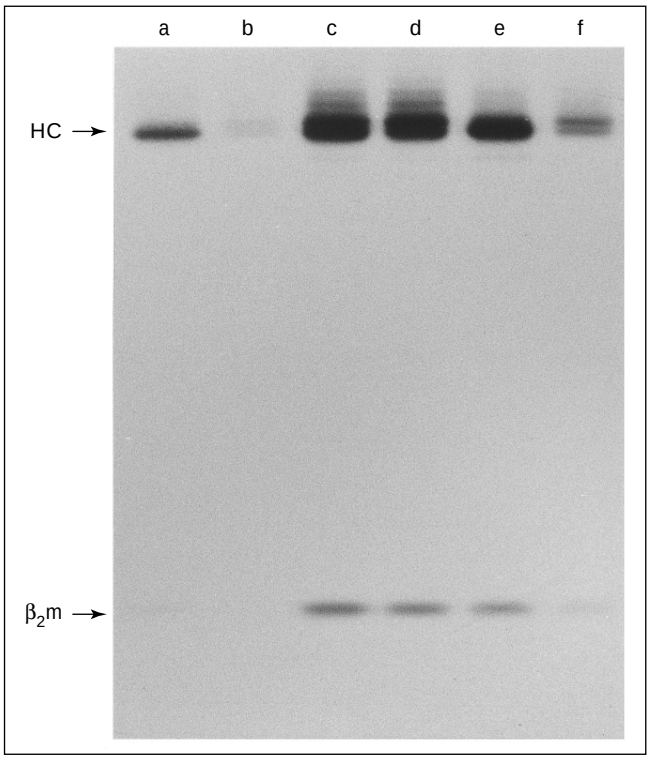
Table 2

Cell-surface expression of wild-type and T134K HLA-A2.1 by C1R transfectants.

	Control antibody*	BB7.2	MA2.1
C1R	15†	20	20
C1R-A2.1	9	898	940
C1R-T134K	12	214	205
.174	10	292	315

*B22.249, an IgG2a monoclonal antibody recognizing H2-Db; †mean fluorescence intensity.

Figure 1



Most T134K molecules are unstable in C1R cells. Immunoprecipitation of class I molecules with BB7.2 from metabolically labelled .174 (lanes a and b), C1R-A2.1 (lanes c and d) and C1R-T134K cells (lanes e and f), following addition of a saturating concentration (10 mM) of the A2.1-stabilizing peptide ILKEPVHGV (lanes a, c and e) or PBS (lanes b, d and f). Note that the ratio of intensity of heavy chain (HC) to β_2m is the same in C1R-A2.1 (lane c) and C1R-T134K (lane e) cells, indicating that the stochiometry of HC–β_2m complexes is the same for both wild-type and mutant class I molecules.

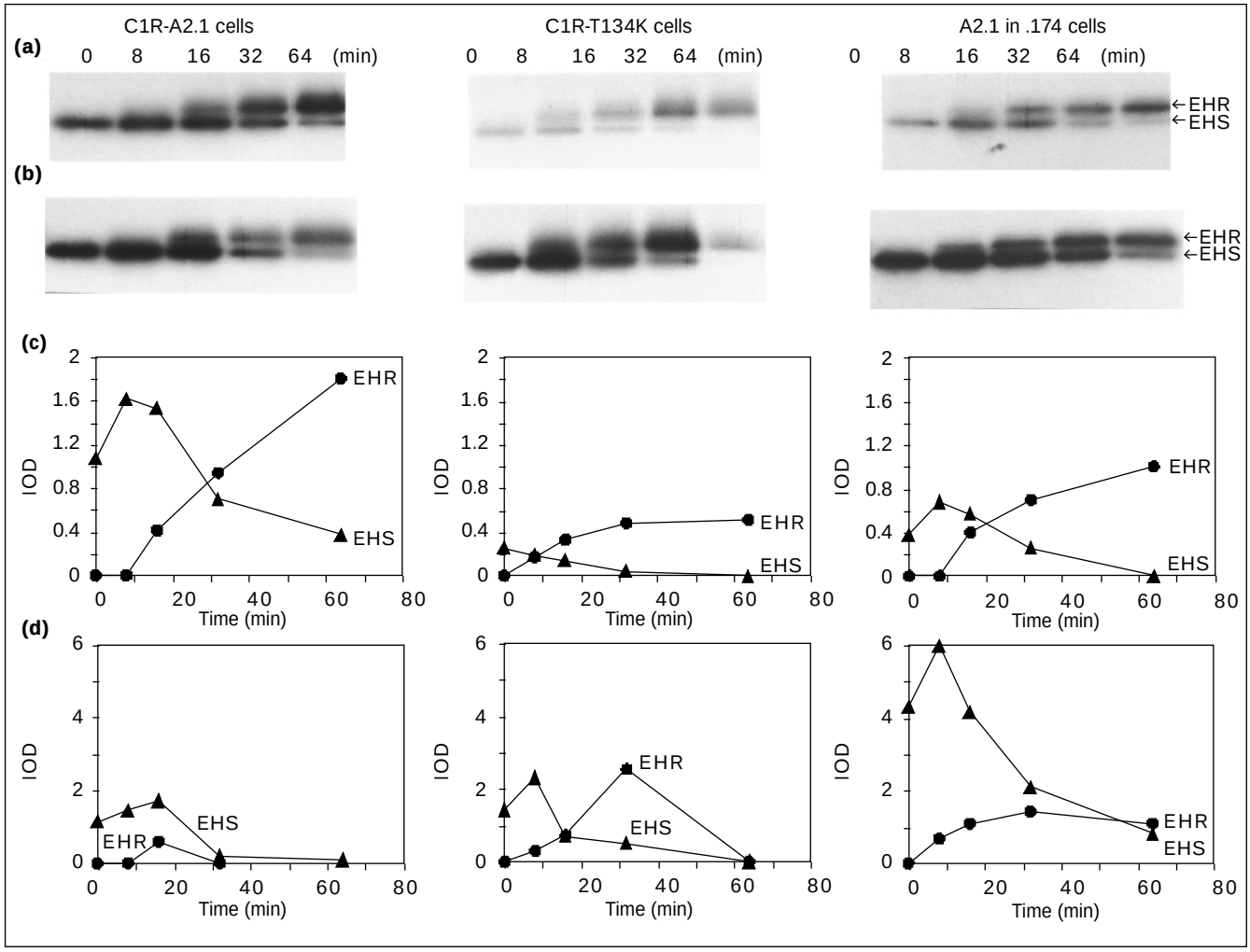
A2.1 molecules recovered from lysates lacking added peptide represent those that assemble and mature as stable heterodimers. The population of A2.1 molecules that would normally fall apart during an overnight incubation in the absence of peptide can be preserved by the addition of stabilizing peptide. This technique allowed us to measure the intracellular fate of both stable and unstable class I molecules during a 1-hour chase.

Unlike an earlier report [20] indicating that the threonine-to-lysine mutation in A2.1 does not affect its rate of intracellular trafficking, we found that in C1R cells, T134K acquires endo-H resistance with a half-time of 12 minutes, which is considerably faster than wild-type A2.1, which matures with a half-time of around 30 minutes (Fig. 2). In C1R cells, around 50 % of wild-type A2.1 molecules seem to be stabilized rapidly. In five separate experiments, this proportion ranged from 50 to 70 %. This is consistent with previous studies, which show that HC–β2m heterodimers are stabilized within 2 minutes of their synthesis. The remaining molecules are apparently degraded in an endo-H-sensitive compartment. A stark contrast is observed with T134K in C1R cells. In this case, although a small proportion (around 25 %) of T134K molecules mature as stable complexes, the majority enter the secretory pathway as unstable heterodimers — they are recoverable only from lysates to which the A2.1-binding peptide has been added. These unstable molecules were released from the ER and, having acquired endo-H resistance, disappeared over the next 30 minutes (compare t = 32 min and t = 64 min in Fig. 2d). Thus, in C1R cells, all T134K molecules mature rapidly, and the majority of these are detectable only as unstable complexes which seem to be peptide-receptive.

The biogenesis of T134K molecules in C1R cells (Fig. 2) has some similarities with the behaviour of wild-type A2.1 molecules in the TAP-negative cell line .174 in that, here too, a significant proportion of A2.1 synthesized enters the secretory pathway as unstable complexes. However, unlike unstable T134K molecules, which are transported much more rapidly, these acquire endo-H resistance with a half-time comparable to wild-type A2.1 in C1R cells (30 minutes). In longer pulse–chase experiments (data not shown) these unstable endo-H-resistant wild-type molecules were also seen to disappear over a period of around 30 minutes — presumably indicating their clearance from the cell surface.

A small fraction of wild-type molecules in .174 cells and T134K molecules in C1R cells do, however, assemble as stable molecules, mature and can be detected at the cell surface by FACS staining. As a result of work using the TAP-negative cell line T2, it has been suggested that these stable molecules represent wild-type A2.1 that binds to peptides delivered to the ER in a TAP-independent way [24,25]. To determine whether this was also the case for T134K in C1R cells, we eluted the peptides from T134K immunopurified from 1×10^{10} cells. The most abundant peptide isolated in this way from T134K had the sequence LLDVPTAAV, derived from the IP-30 signal sequence and which has been identified previously as the most abundant peptide associated with wild-type A2.1 molecules in a TAP-negative cell line [24,25]. No peptides derived from cytosolic proteins were found associated with T134K. In contrast, the most abundant peptide eluted from wild-type A2.1 in control experiments was derived from the cytosolic 60S ribosomal protein. No signal-sequence-derived peptides were found associated with wild-type A2.1 in these experiments (J.W.L., unpublished observations). The main differences between wild-type A2.1 in .174 and T134K in C1R cells are therefore: the rate of trafficking of both stable and unstable molecules; and the fact that the majority of wild-type molecules in .174 never enter the secretory pathway, whereas in T134K the majority leave the ER and mature as unstable molecules.

Figure 2



T134K molecules are transported rapidly to the cell surface as empty receptors. The intracellular trafficking of wild-type (C1R-A2.1 and .174) and mutant (C1R-T134K) A2.1 molecules. Pulse–chase analysis of a cohort of A2.1 molecules was carried out and BB7.2-reactive molecules were recovered from cell lysates after overnight preclearing in the absence (a) or presence (b) of a saturating concentration (15 mM) of stabilizing peptide. Only the heavy-chain bands are shown, as human β_2m is poorly visualized following a 10-minute pulse label. (c) Quantitation of (a), representing the maturation of stably assembled A2.1. (d) The fate of unstable A2.1 molecules, calculated from the difference in band intensities between (a) and (b) (d = b–a). EHR: endo-H resistant. EHS: endo-H sensitive. IOD: integrated optical density in arbitrary units (see Materials and methods). All IODs were normalized to the amount of immune complex recovered at the end of the experiment. This was achieved by comparing the relative intensity of the Coomassie blue-stained monoclonal antibody heavy-chain band observed before autoradiography.

The T134K heavy chain binds both peptide and β_2m normally

One explanation for our observation that in TAP-competent cells most T134K assembles as unstable molecules is that the mutation leads to an inherent inability to bind peptide and β_2m normally. First, we measured the ability of three different A2.1-binding peptides to stabilize 'empty' wild-type A2.1 (expressed in .174 cells) and T134K (expressed in C1R cells). This has been shown previously to be a reasonable estimate of the affinity of peptide-binding at equilibrium [26]. Binding was dose-dependent in all cases, and showed half-maximal effects at comparable concentrations for both wild-type A2.1 and T134K, as shown in Table 3. We also showed there was no difference in the binding of peptides to immature (endo-H-sensitive) *versus* mature (endo-H-resistant) class I molecules, for wild-type or T134K (Table 3). This is important, as it shows there is no destabilizing effect specific for the T134K mutation of sialic-acid-containing complex sugars. In addition, the rate of dissociation of one of the peptides (radiolabelled ILKEPVHGV) was shown to be the same for both wild-type and T134K, indicating that the threonine-to-lysine mutation did not significantly alter the binding kinetics of the A2.1 molecule, and that T134K was able to form very stable complexes with peptide.

Table 3

The binding of three peptides to wild-type A2.1 and T134K.

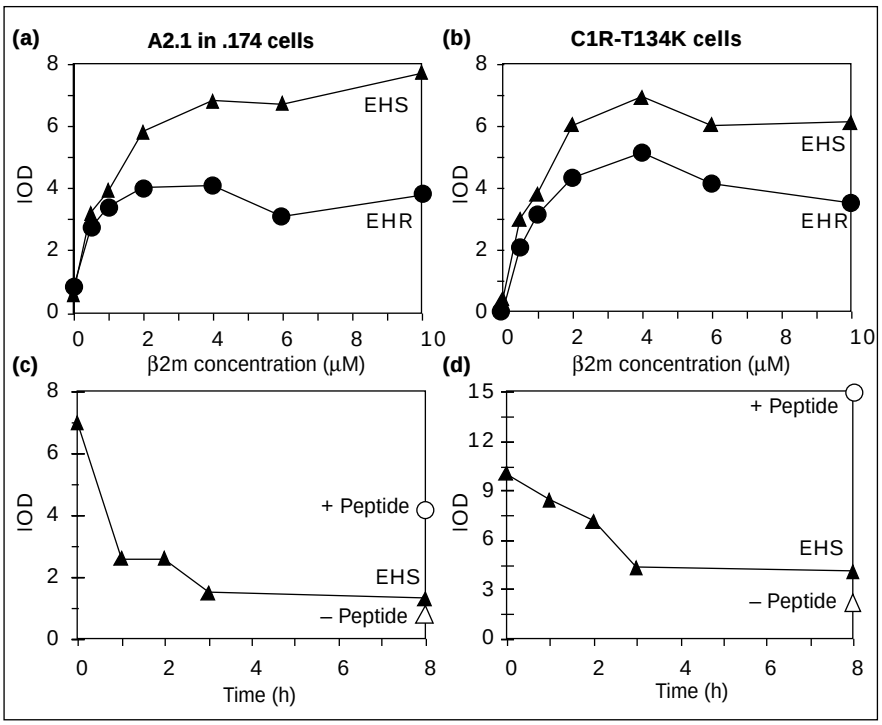
	Wild-type A2.1			T134K		
	Kd* (nM) mature†	Kd (nM) immature‡	t½§ (h)	Kd (nM) mature	Kd (nM) immature	t½ (9 h)
GILGFVFTL	3	5	ND	6	8	ND
ILKEPVHGV	8	5	220	10	8	260
TLWVDPYEV	50	50	ND	70	70	ND

*An estimation of the concentration of peptide required to effect half-maximal stabilization of class I molecules in a cell lysate was taken as a rough estimate of binding at equilibrium (Kd). †Mature molecules are those which are endo-H insensitive. ‡Immature molecules are those which are endo-H sensitive. §Half-time for dissociation.

Second, we compared the intrinsic ability of wild-type A2.1 and T134K heavy chains to assemble with human β_2m. As shown in Figure 3a,b, the assembly of class I molecules in dilute lysates containing unstable HC–β_2m heterodimers can be driven by raising the concentration of free β_2m. Again, assembly is dose-dependent, and seems to proceed according to the law of mass action. The half-maximal concentration of β_2m required for the effect is around 0.5 μM for both wild-type A2.1 and T134K (in both endo-H-sensitive and endo-H-resistant forms), which indicates that the binding affinities of β_2m to these heavy-chains in the absence of peptide are the same. This is also supported by the fact that we did not observe any difference in the degree of reactivity with the W6/32 antibody (which depends on β_2m for recognition of HLA molecules) when we used it to precipitate wild-type and mutant molecules from digitonin lysates (see, for example, Fig. 4).

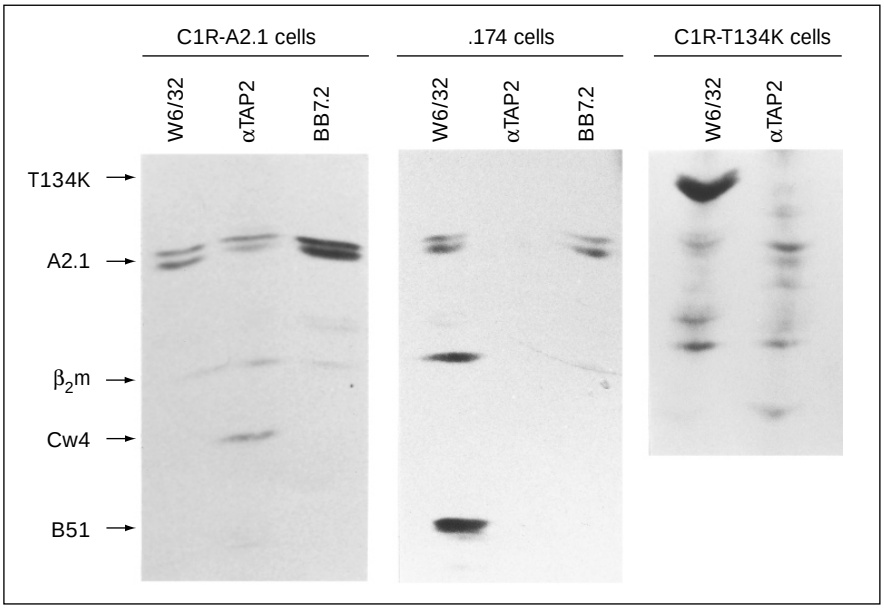
The binding of peptides to HC–β_2m increases the affinity of HC for β_2m by more than 10-fold [10]. The failure of peptide and β_2m to bind co-operatively in this way to T134K could be another explanation for the observation that most T134K seems to assemble as unstable complexes with β_2m. To assess the ability of peptide to stabilize T134K–β_2m heterodimers, we measured the rate of decay of assembled T134K in the presence and absence of peptide. We have previously shown that the loss of a conformation-sensitive epitope on the α1/α2 domains of HC correlates extremely well with the loss of β_2m from the complex

Figure 3



T134K assembles normally with β_2m in cell lysates. The dose-dependent binding and stabilization of wild-type (a) and T134K (b) A2.1 with human β_2m was measured for both endo-H-sensitive (EHS) and endo-H-resistant (EHR) class I molecules. The rate of dissociation and subsequent loss of the BB7.2 epitope was also measured for wild-type (c) and T134K (d) A2.1 in absence of a saturating concentration of stabilizing peptide. Open symbols represent the number of BB7.2-reactive complexes precipitable after 20 h in the presence or absence of peptide.

Figure 4



T134K does not associate with the TAP1–TAP2 heterodimer in C1R cells. Metabolically labelled lysates of C1R-A2.1, .174 and C1R-T134K cells were immunoprecipitated with monoclonal antibody W6/32 or anti-TAP2 antiserum. In addition, monoclonal antibody BB7.2 was used to immunoprecipitate wild-type A2.1 from C1R-A2.1 and .174 cell lysates. The immunoprecipitates were analysed by 1D-IEF to resolve co-precipitating A2.1 and Cw4. The data shown for C1R-A2.1 and .174 cells, and C1R-T134K cells, were from separate experiments. Note, W6/32 also precipitates endogenous HLA-B51 (B51) expressed in .174 cells.

[10]. We therefore measured the rate of decay of total T134K–β_2m heterodimers (this includes a mixture of both mature and immature molecules) using this technique. Figure 3c,d shows that the rate of decay of T134K has a half-time of around 2.5 hours in the absence of peptide, and more than 20 hours in the presence of peptide, and is consistent with the equivalent rates of decay of wild-type A2.1 assembled in .174 cells. It seems, therefore, that the mutation at position 134 does not alter the intrinsic ability of the A2.1 molecule to assemble normally with β_2m and peptides, to give rise to a long-lived heterotrimeric complex *in vitro*.

To show that the same was true *in vivo*, we delivered a 'pre-processed' CTL epitope directly to the ER of C1R-T134K cells. TAP-independent delivery of the influenza A matrix protein (MP) epitope GILGFVFTL is optimized by delivering it in the form of a minigene preceded by an ER translocation signal sequence [25,27]. Table 4 shows that delivering the epitope in this way overcomes the antigen-presentation defect seen with full-length MP in both C1R-T134K and .174 cells. Influenza A MP is clearly able to be processed and delivered to the ER of C1R cells, as shown by the ability of wild-type A2.1 to present this epitope in C1R cells infected with influenza A/X31 (Table 4).

T134K does not associate with the TAP1–TAP2 heterodimer in C1R cells

Having established that the intrinsic properties of the mutant A2.1 molecule are normal, we concluded that the differences we observed between T134K and wild-type A2.1 expressed in C1R cells must be due to the disruption of an intracellular process which acts on the newly synthesized class I molecules, enabling them to assemble stably with cytosolically derived peptides. We wondered, therefore, whether the mutation at position 134 disrupted an interaction between the class I molecule and the TAP1–TAP2 heterodimer, leading to an inability to access peptides that are delivered there by the transporter. This might be expected to produce a block in the antigen-presentation pathway at the same stage as is seen in .174 cells, where TAP1–TAP2 is absent, resulting in the assembly of unstable class I molecules. Figure 4 shows that this is indeed the case. Whereas wild-type A2.1 and HLA-Cw4 could be recovered in association with TAP, no TAP-associated T134K could be detected. Note also that T134K migrates higher than wild-type A2.1 in the IEF

Table 4

Sensitization of C1R-A2.1 and C1R-T134K cells with L+G9L vacc for lysis by CTLs.

	C1R-A2.1	C1R-T134K	.174
10 nM peptide*			
10:1†	61‡	70	78
2:1	52	57	60
WR vacc			
10:1	10	10	6
2:1	7	3	1
Influenza A/X31			
10:1	67	8	9
2:1	39	9	5
L+G9L vacc			
10:1	28	24	38
2:1	17	13	22

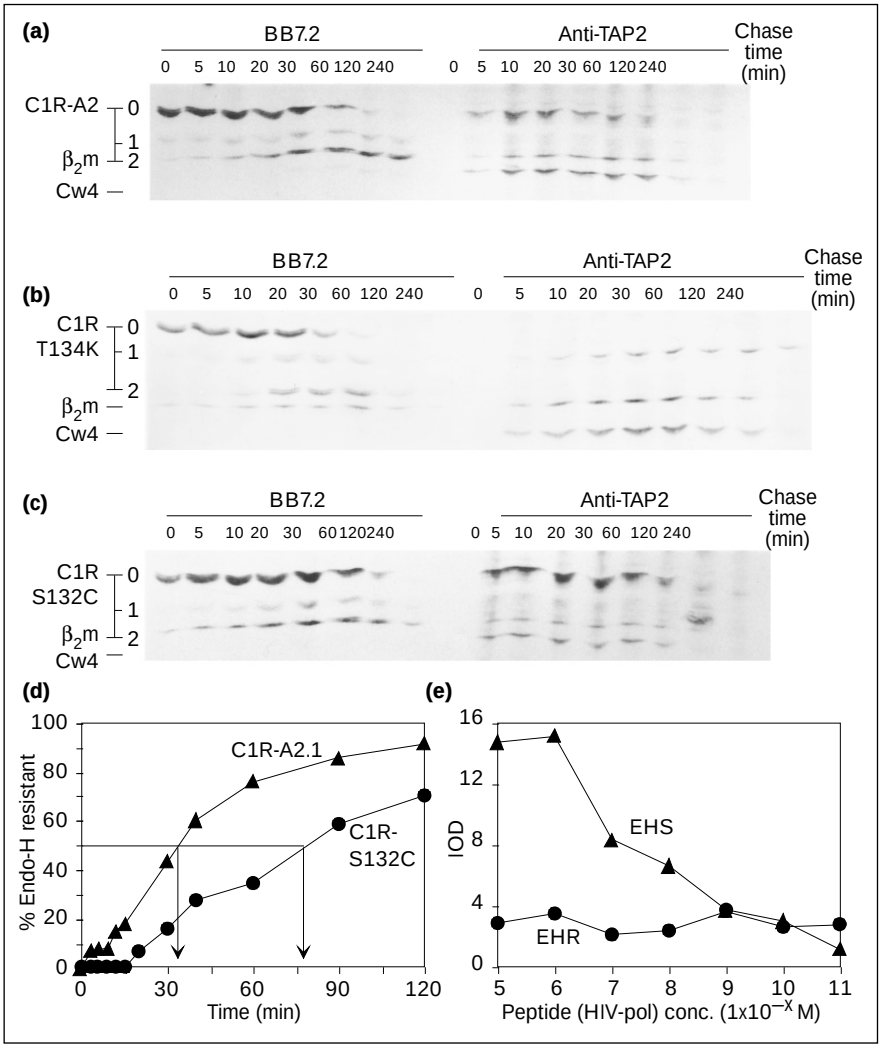
*Influenza A matrix protein 55–63 (GILGFVFTL); †effector to target ratio; ‡percent specific lysis.

gel, which separates proteins according to their isoelectric point (pI). This is due to the addition of positive charge by the threonine-to-lysine mutation. HLA-Cw4 in association with TAP is recovered equally well from lysates of C1R-A2.1 and C1R-T134K cells. We do not know the origin of the additional bands that co-precipitate with the anti-TAP antiserum, nor whether they are specifically co-precipitated. Only the band that equilibrates at a pI similar to that of mono-sialylated T134K was reproducibly co-precipitated with anti-TAP (see, for example, Fig. 6). This band is unlikely to be mono- or non-sialylated T134K heavy chain because: it equilibrates at a different pI to either of these; A2.1 in all its sialylated forms migrates as a doublet, but this band is a singlet; the band persists after the β_2m band disappears, indicating that, unlike Cw4 in the same cell, it is not associated with β_2m, as might be expected if it were T134K; and, sialylation occurs in the *trans*-Golgi network whereas TAP localizes to the ER and *cis*-Golgi network [28].

Association between TAP binding and trafficking rate of class I complexes

The lack of TAP association could explain why the intracellular loading of T134K is defective, but does not obviously account for why unstable (empty) molecules are transported rapidly to the cell surface. One explanation may be that TAP acts with a chaperonin-like function to retain class I molecules in the ER until they are properly folded. Indeed, Figure 5a shows that in C1R cells, the release of wild-type molecules from TAP and their rate of maturation (acquisition of sialic acids) are co-ordinated with a half-time of around 30 minutes. Figure 5b also shows that T134K does not associate with TAP at any time during its rapid maturation. These data are consistent with observations made with other HLA class I alleles, where the rate of intracellular trafficking correlates well with the amount of that class I allele found bound to TAP in immunoprecipitates ([29] and A.N., unpublished observations). To investigate this relationship in more detail, we examined the

Figure 5



TAP association and trafficking rate may be linked phenomena. Pulse–chase analysis of a cohort of A2.1 molecules was carried out, by precipitating digitonin lysates of C1R-A2.1 (a), C1R-T134K (b) and C1R-S132C cells (c) with either BB7.2 (left) or anti-TAP2 antiserum (right). HLA-A2.1 carrying no (immature), one or two (mature) sialic acid residues are labelled 0, 1 and 2 correspondingly. The autoradiographic exposure we have chosen to show here, although optimal for illustrating TAP co-precipitation, is not optimized to demonstrate the difference in trafficking between wild-type A2.1 and S132C. This is shown better in (d), where the trafficking rates of S132C and wild-type A2.1 in C1R cells were measured using the pulse–chase method described for Figure 2 . (e) Very few mature (endo-H resistant; EHR) S132C molecules are peptide-receptive, although immature (endo-H sensitive; EHS) S132C molecules bind the HIV-pol peptide in a dose-dependent way similar to wild-type A2.1 and T134K molecules.

biogenesis of a second point mutant, in which serine at position 132 is substituted for cysteine (S132C). This mutant presents endogenous antigen efficiently, but exogenous peptides very poorly [20]. S132C is transported very slowly in C1R cells, having a half-time for maturation of around 85 minutes (Fig. 5d). Significantly, it associates with TAP for much of this time (Fig. 5c) and results in the egress of stably assembled complexes. Figure 5e shows that no S132C molecules leave the ER in a peptide-receptive state, as the addition of peptides to endo-H-resistant molecules did not increase the number of complexes recovered by BB7.2. There are sufficient numbers of immature S132C molecules, however, to show that S132C binds peptide normally in a lysate (half-maximal effect at around 1×10^{-8} M, compared to 0.5×10^{-8} M for wild-type A2.1 and 0.8×10^{-8} M for T134K, see Table 3). Thus, the biochemistry of S132C is consistent with its antigen-presentation phenotype, and strongly supports the notion that the duration of TAP association is directly correlated with the number or proportion of class I molecules that leave the ER loaded with an optimal peptide. S132C and T134K may therefore represent two ends of a spectrum along which all class I molecules fall.

Discussion

Our results show that a single amino-acid substitution at position 134 in the α2 domain of the HLA-A2.1. HC (T134K) results in the intracellular assembly of unstable complexes with β_2m, which are transported to the cell surface more than three times faster than the wild-type A2.1 molecules expressed in the same cell. The mutant molecules can bind to peptides and β_2m normally *in vitro*. However, no detectable association between the mutant HC and the TAP1–TAP2 heterodimer was found in co-immunoprecipitation experiments. The most likely explanation to account for the observed behaviour of the mutant molecules, therefore, is that they fail to become loaded with high-affinity peptides in the lumen of the ER as a consequence of this impaired association. This is the first demonstration that the MHC class I–TAP interaction is obligatory for the presentation of peptide epitopes delivered to the ER by TAP. These results are consistent with the functional phenotype of C1R-T134K cells, which does not present endogenous antigen to CTLs, but presents exogenously added peptides normally.

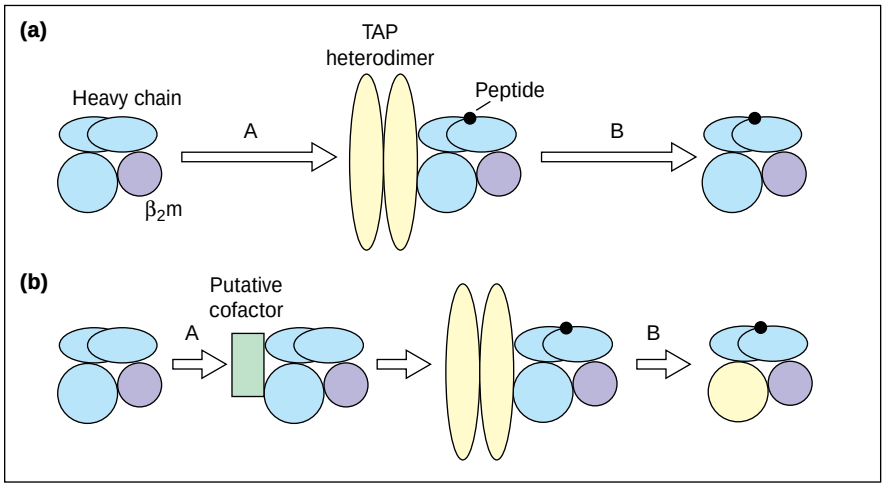
It remains to be seen whether the requirement for TAP association that we observe for HLA-A2.1 can be extended to other class I molecules. Although the behaviour of the mutant heavy chains could be a consequence of studying them in the cell line C1R (which has its origins in a γ-irradiated EBV-transformed B-cell line [30]), it is of interest to note that Neisig *et al.* [29] have found other naturally occurring HLA class I alleles that are capable of presenting antigen, but that bind only poorly or undetectably to TAP. The only difference among HLA alleles in the region between residues 128 and 137 is an arginine/serine dimorphism at position 131. However, alleles with both arginine and serine are represented among both efficient and inefficient TAP associators. Also, a recent report by Lee *et al.* [31] showed that a soluble form of HLA-G loaded the same set of endogenous peptides as its membrane-anchored counterpart, despite a lack of demonstrable TAP association.

The behaviour of the T134K molecule in being unable to form stable complexes with peptides in TAP-competent cells is similar to that of another class I molecule, D^{dm6} [32], which has a non-conservative amino-acid substitution within the same highly conserved region of the α2 domain between residues 128 and 137 (the loop preceding, and most of, the β4 strand). In all classical and non-classical murine class I molecules, this sequence is EDLK-TWTAAD (the human equivalent is EDLRSWTAAD). In D^{dm6}, residue 133 is changed from tryptophan to arginine (W133R), the only different residue between the D^d and D^{dm6} class I molecules. The level of cell-surface expression of the mutant in normal cells is less than 10 % of the levels of K^d or D^d expression in the same cells, and D^{dm6} could only be precipitated from pre-cleared lysates with antibodies to the α3 domain, and not to the α1/α2 domains. Nevertheless, the mutant heavy chains were shown to acquire mature N-linked oligosaccharides. Although experiments were not carried out to test whether D^{dm6} could be stabilized with D^d-binding peptides, or whether it was able to present D^d-restricted endogenous antigens, it is tempting to speculate that the mutant molecules behave like T134K.

Taken together with our observations that a threonine-to-lysine substitution at position 134 ablates the class I interaction with TAP, and that another mutation in this region (S132C) results in slow trafficking and a phenotype consistent with 'hyper-efficient' intracellular loading of peptides, we suggest that residues in this region of the molecule are responsible for dictating the efficiency of loading by controlling the interaction of HC with TAP, either directly or indirectly. Recent reports have also implicated residues in both the α3 domain [33] and the peptide-binding groove [29] in controlling the efficacy of TAP binding. It remains to be seen whether these observations, along with our observation that solvent-exposed residues in the α2 domain are also involved, will result in an unified description of the class I–TAP interaction.

Various models can be proposed for the *in vivo* function of TAP in the biogenesis of stable class I–peptide complexes and their regulated release into the anteriograde transport pathway. All of these must accommodate the observed behaviour of T134K in CIR cells. The simplest is that the conserved stretch between residues 128 and 137 forms all or part of the interface between class I and TAP, and that TAP has a chaperonin function, retaining class I heterodimers in the ER until a suitable peptide for binding is

Figure 6



Models to account for the intracellular role of TAP in regulating the assembly of stable class I–peptide trimeric complexes. (a) Newly assembled HC–β_2m complexes associate with TAP, where they acquire stabilizing ligands and are subsequently released. Release of stabilized HC–β_2m–peptide complexes is the rate-determining step here. (b) Alternatively, newly assembled complexes could bind to a molecule which either chaperones them to TAP or acts as a cofactor for TAP binding and which rescues empty molecules from the anteriograde transport pathway until they acquire stably bound peptide ligands, whereupon they are released. A: step blocked by T134K mutation. B: rate-limiting step.

delivered (Fig. 6a). The T134K mutation may destabilize this interface, resulting in the early entry of unloaded class I molecules into the secretory pathway. This model, however, would predict that wild-type A2.1 in a TAP-negative cell line should traffic rapidly, which it does not. Variations of this model therefore invoke an accessory molecule which could bind to empty class I molecules and either conduct them to TAP or be otherwise required for the formation of an MHC class I–TAP complex (Fig. 6b). The rate-limiting release of loaded class I molecules from the HC–TAP complex might be controlled by TAP or by the putative cofactor. Failure to associate with this factor would bypass the rate-determining step and result in the premature release of unloaded, unstable molecules into the anteriograde transport pathway, as is the case for T134K. Potential candidates for such a factor include the ER chaperones calnexin and calreticulin, as well as the component that is presumed to be missing in the recently described mutant cell line .220 [34]. In .220 cells, two transfected HLA class I alleles display defective assembly as a result of their impaired interaction with TAP [35]. This model accommodates the observation that in the TAP-negative cell line .174 (which might be expected to express the accessory molecule), wild-type A2.1 is transported slowly, and predicts that the rapid transport of T134K might be TAP-independent and independent of peptide binding in the ER. To test this, we are currently investigating whether the rate of transport for T134K and S132C is TAP-dependent.

Materials and methods

Cells and antibodies

The human B-lymphoblastoid cell line C1R, a derivative of Hym2 [36], was transfected with plasmids encoding the genes for wild-type A2.1, A2.1 with a single coding change resulting in a threonine to lysine substitution at position 134 (T134K), and a similar point mutation resulting in a serine to cysteine substitution at position 132 (S132C), by M. Matsui (Duke University; [20]) and were a kind gift from J. Frelinger. Cell lines were cultured in RPMI 1640 cell culture medium (Gibco Laboratories) supplemented with 10 % FCS, 2 mM L-glutamine, and penicillin/streptomycin (Gibco BRL) at 37 °C; 5 % CO_2. The C1R cell line, which has one copy of chromosome 6, does not express endogenous HLA-A or HLA-B but does express endogenous Cw4 [30]. The transporter-deficient cell line LBL 721.174 (.174) was kindly provided by R. DeMars. An HLA-A2.1-restricted CTL line recognizing the influenza A matrix protein epitope GILGFVFTL was a gift from V. Braud (University of Oxford), and the A2.1-restricted anti-HIV-pol epitope ILKEPVHGV was a gift from A. McMichael (University of Oxford).

Rabbit anti-TAP2 serum was made by constructing a GST fusion protein containing the ATP-binding domain of TAP2 (amino acids 507–798). Templates for PCR were a generous gift from J. Trowsdale (ICRF, London) and the fragments were cloned in the pGEX vector. The GST-fusion proteins were over-expressed in *E. coli*, isolated as inclusion bodies by differential centrifugation and injected into rabbits. The monoclonal antibodies used were anti-HLA-A2.1 (BB7.2 [37]) and anti-HLA-A, HLA-B, HLA-C (W6/32 [38]).

Peptide synthesis

All peptides were synthesized by solid-phase syntheses using fmoc chemistry, cleaved with TFA and purified to more than 95 % homogeneity by reversed-phase HPLC. Peptides used in this investigation were: GILGFVFTL (influenza A matrix protein residues 55–63, [39]); ILKEPVHGV (HIV-pol residues 476–484, [40]) and TLWVDPYEV (unidentified self peptide eluted from HLA-A2.1 [41]).

Cell-surface expression of MHC class I detected by flow cytometry

Cells (1×10^6) were washed into PBS containing 0.1 % BSA before adding a saturating concentration of monoclonal antibody. After 30 min at 4 °C, cells were washed three times in PBS/BSA, then resuspended in a saturating concentration of fluorescein-conjugated goat anti-mouse IgG (Sigma, St Louis). The cells were incubated for 30 min at 4 °C, washed three times, resuspended in PBS and analysed by FACS. Each sample analysed comprized a minimum of 5×10^4 viable cells.

Metabolic labelling, pulse–chase, and immunoprecipitations of class I molecules

Cells (1×10^7 per experimental point) were washed in PBS and starved for 40 min in 5 ml methionine/cysteine-free RPMI 1640 media, containing 10 % dialysed FCS. After labelling cells for 10 min with 100 μCi [^{35}S] methionine/cysteine trans-label (Amersham) per 10^7 cells at 37 °C, they were chased for 0, 8, 16, 32, 64 min in RPMI 1640 medium supplemented with 200 mM methionine/cysteine and 10 % FCS. At each time point, cell aliquots were removed, washed in ice-cold PBS and lysed in 1 ml ice-cold

Tris lysis buffer (pH 7.4), containing 0.5 % NP-40 and 0.5 % Mega-9, 5 mM iodoacetamide and 2 mM PMSF (Sigma). For aliquots lysed in the presence of stabilizing peptide, 15 μM HIV-pol(476–484) was added to the lysis mix. After 15 min at 4 °C, the nuclei were removed by centrifugation and the supernatants were precleared overnight at 4 °C with 20 μl of fixed *Staphylococcus aureus* (Sigma) made up in lysis buffer.

Assembled class I molecules were immunoprecipitated with BB7.2 (10 μg ml^{-1} for 1 h) and 5 % (w/w) protein A–Sepharose beads (Sigma). After washing the immunoprecipitates three times in lysis buffer, they were subjected to endoglycosidase-H digestion overnight before being analysed on a 10 % SDS–PAGE gel under reducing conditions. Gels were fixed, stained, treated with Amplify (Amersham), dried and visualized by autoradiography at –80 °C. Quantitation of autoradiograms was achieved by scanning using a MilliGen Bioimage Analyser which expresses band intensities as an integrated optical density (IOD).

Endoglycosidase-H treatment

Endoglycosidase-H (Boehringer Mannheim) digestion of immunoprecipitates was performed by resuspending the samples in 30 μl of 50 mM sodium citrate buffer (pH 5.5), containing 0.2 % SDS, heating to 95 °C for 5 min, and digesting with 3 U of endoglycosidase-H overnight at 37 °C.

Peptide or β_2m binding in vitro

Increasing concentrations of β_2m or stabilizing peptide were added to cell lysates to determine binding affinities for mutant and wild-type class I molecules. Cells were labelled for 15 min with [^{35}S] methionine/cysteine as described above. To enable the determination of binding affinities for both endoglycosidase-H-resistant (EHR) and endoglycosidase-H-sensitive (EHS) forms, each cell line was chased with cold RPMI 1640 medium supplemented with 200 mM methionine/cysteine and 10 % FCS (as above) until equal amounts of both EHR and EHS class I molecules were obtained. (For pulse–chase experiments above, C1R and .174 cells were chased for 30 min, C1R-T134K cells were chased for 12 min, and C1R-S132C cells were chased for 60 min.) After washing cells in cold PBS, samples were lysed in ice-cold Tris lysis buffer (as above) in the presence of a decreasing concentration of either β_2m or peptide. Samples were then precleared for 1 h with Staph A and immunoprecipitated using BB7.2, washed three times, exposed to endoglycosidase-H overnight and analysed as above by 12 % SDS–PAGE.

β_2m dissociation assay

The stability of wild-type A2 and T134K class I complexes was determined *in vitro* by measuring the rate of decay of the BB7.2 epitope resulting from the dissociation of β_2m from HC. A2.1 molecules in .174 and C1R-T134K cells were labelled with [^{35}S] methionine/cysteine and chased as described previously to obtain equal amounts of EHS and EHR class I molecules. Cells were then lysed in Tris lysis buffer containing 0.5 % NP-40, aliquotted and kept on ice. At intervals following lysis (0, 1, 2, 3, 8 and 20 h), stabilizing HIV-pol peptide and BB7.2 antibody were added together to the lysate and immunoprecipitated after 1 h (as above). For one sample, peptide was added at the time of lysis (0 h) and BB7.2 was added at 20 h. Samples were analysed on SDS–PAGE after endoglycosidase-H treatment (as above).

Association of class I molecules with TAP

We starved 2×10^7 cells of each cell line for 40 min in 1 ml methionine/cysteine-free RPMI 1640 medium supplemented with 10 % FCS. The cells were pelletted and resuspended in 500 μl fresh methionine/cysteine-free RPMI 1640 + 10 % FCS and labelled with 250 μCi ^{35}S-methionine/cysteine for 10 min at 37 °C. Cells were washed twice with cold PBS and lysed in 500 μl 1 % digitonin-containing lysis buffer (1 % (w/v) digitonin (Sigma), 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 150 mM NaCl) for 30 min on ice. Each lysate was split into equal portions, pre-cleared overnight with normal rabbit serum (NRS) and immunoprecipitated with either BB7.2, W6/32 or anti-TAP2 antibodies. The immunoprecipitations were recovered with protein A–Sepharose beads (Sigma) for 1 h and the beads were washed three times in lysis buffer containing 0.5 % digitonin before analysis by 1D-IEF.

Pulse–chase analysis to follow assembly of class I molecules and TAP–class I interactions

We starved 1×10^8 cells for 40 min in 2 ml methionine/cysteine-free RPMI 1640 medium with 10 % FCS. Cells were pelletted, resuspended in 500 μl fresh RPMI 1640 medium supplemented with 10 % FCS and labelled with 500 μCi ^{35}S-methionine/cysteine for 10 min at 37 °C. Cells were chased for 0, 5, 10, 20, 30, 60, 120, 240 and 480 min in RPMI 1640 medium supplemented with 1 mM methionine/cysteine and 10 % FCS. Cells were washed in ice-cold PBS before lysis in 1 % digitonin-containing lysis buffer for 1 h on ice. Each lysate was divided into two portions, pre-cleared with NRS and immunoprecipitated with either BB7.2 or anti-TAP2 antibodies. All samples were analysed by 1D-IEF.

One-dimensional isoelectric focusing (1D-IEF)

1D-IEF was performed as described [42].

Dissociation rate of peptide from class I

The peptide ILKEVPHGV was radio-iodinated to a specific activity of 1.3×10^{16} cpm mol^{-1} using the chloramine T method. C1R, .174 and C1R-T134K cells (1×10^8) were lysed in 1 ml cold lysis buffer and nuclei were removed by centrifugation. Peptide (5×10^7 cpm) was then added to each sample along with human β_2m and diluted to a final concentration of 2×10^7 cell equivalents per ml and 3 mg ml^{-1} β_2m. This mixture was allowed to equilibrate overnight at 4 °C. The next day the equilibrium mixture was diluted 1:1 with a 0.2 mM solution of unlabelled ILKEPVHGV in cold lysis buffer, thus effecting a 100-fold molar excess of cold over hot. At various times during the following 300 h, 2×10^7 cell equivalents were removed and precipitated with BB7.2, as described above. The immunoprecipitates were washed four times and the resulting isolates were quantitated with a Rackgamma γ-counter (LKB). Background binding was determined by allowing 2×10^7 cell equivalents of lysate to equilibrate with 5×10^6 cpm hot ILKEPVHGV in a solution of 0.1 mM cold peptide. Specific peptide-binding to wild-type A2.1 and T134K were calculated by subtracting background cpm obtained in the sham binding control with a lysate of untransfected C1R cells.

Construction of recombinant vaccinia virus and CTL assays

Recombinant vaccinia virus expressing a minigene encoding the optimal influenza A matrix protein epitope GILGFVFTL preceded by the haemagglutinin ER translocation signal sequence MKANLLVLLCALAAADA (L+G9L-vacc) was constructed as described in [8]. Briefly, the synthetic minigene was cloned into the plasmid pSC11.302.R.L+ as an *Nco*I–*Bam*HI fragment. The resulting construct was then introduced into the thymidine kinase locus of WR vaccinia by homologous recombination using standard techniques.

The ability of recombinant vaccinias to sensitize C1R-A2.1 and C1R-T134K cells was assessed by infecting 1×10^6 of each for 1 h during ^{51}Cr-labelling at a multiplicity of infection of 20. Influenza A/X31 infections were with 200 μl allantoic fluid. The media with virus and ^{51}Cr were then washed away and the cells were incubated for an additional 2 h at 37 °C. Following two further washes in R10, the target cells were assayed for their susceptibility to lysis by CTLs in a 4-hour chromium-release assay. Where peptide sensitization was assayed, targets were pulsed with 10 nM peptide 1 h prior to the start of the CTL assay.

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