Surface expression, peptide repertoire, and thermostability of chicken class I molecules correlate with peptide transporter specificity

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The chicken major histocompatibility complex (MHC) has strong genetic associations with resistance and susceptibility to certain infectious pathogens. The cell surface expression level of MHC class I molecules varies as much as 10-fold between chicken haplotypes and is inversely correlated with diversity of peptide repertoire and with resistance to Marek's disease caused by an oncogenic herpesvirus. Here we show that the average thermostability of class I molecules isolated from cells also varies, being higher for high-expressing MHC haplotypes. However, we find roughly the same amount of class I protein synthesized by high- and low-expressing MHC haplotypes, with movement to the cell surface responsible for the difference in expression. Previous data show that chicken TAP genes have high allelic polymorphism, with peptide translocation specific for each MHC haplotype. Here we use assembly assays with peptide libraries to show that high-expressing B15 class I molecules can bind a much wider variety of peptides than are found on the cell surface, with the B15 TAPs restricting the peptides available. In contrast, the translocation specificity of TAPs from the low-expressing B21 haplotype is even more permissive than the promiscuous binding shown by the dominantly expressed class I molecule. B15/B21 heterozygote cells show much greater expression of B15 class I molecules than B15/B15 homozygote cells, presumably as a result of receiving additional peptides from the B21 TAPs. Thus, chicken MHC haplotypes vary in several correlated attributes, with the most obvious candidate linking all these properties being molecular interactions within the peptide-loading complex (PLC).

ABC transporter | restrictive | permissive | heterozygous advantage | overdominance

Classical class I molecules of the major histocompatibility complex (MHC) play crucial roles in the immune response and other biological phenomena, presenting peptides to T lymphocytes as well as being recognized by natural killer cells (1–3). MHC class I molecules have high allelic polymorphism and sequence diversity, with many of the variable positions involved in binding peptides. The general consensus is that this polymorphism is driven by a molecular arms race with infectious pathogens (4, 5).

Class I molecules are also polymorphic in expression at the cell surface, discovered in chickens (6, 7), but more recently found for HLA-C in humans (8, 9). It has also become clear that the diversity of peptides bound by particular class I alleles varies significantly, again described first in chickens (10, 11) and later in humans (12, 13). More recently, we reported that these two properties, cell surface expression and peptide repertoire, are inversely correlated for both chicken class I molecules and four human HLA-B alleles (14). These properties are also associated with resistance to certain infectious pathogens: low-expressing promiscuous molecules with resistance to Marek's disease in chickens and high-expressing fastidious molecules with nonprogression to AIDS in humans (12, 14). On the basis of these findings, we have proposed that class I alleles vary in peptide repertoires to allow different strategies in pathogen

resistance, and vary in expression level for optimization of the peripheral T-cell repertoire (14).

Here we explore the mechanism that leads to the expression level polymorphism in chicken class I molecules. In chickens, unlike mammals, the heterodimeric molecule that pumps peptides from the cytoplasm to the lumen of the endoplasmic reticulum [transporter for antigen presentation (TAP)] and the dedicated chaperone that is involved in peptide editing [tapasin or TAP-binding protein (TAPBP)] both have high allelic polymorphism, moderate sequence diversity, and consequent functional variation (15–17). Coevolution between the TAP and class I genes in chickens leads to the expression of a single, dominantly expressed class I (BF2) gene (10, 11, 16, 18), which can have profound effects on the immune response to infectious pathogens. The data in this report show that thermostability and translocation specificity of the polymorphic TAPs are part of a suite of properties that overall implicate the peptide loading complex (PLC) in determining class I expression level.

Results

High-Expressing Haplotypes Produce Class I Molecules with Higher Thermal Stability than Low-Expressing Haplotypes. One explanation for the difference in cell surface expression between haplotypes

Significance

Major histocompatibility complex (MHC) molecules play crucial roles in the immune response to pathogens and tumors by presenting protein fragments (peptides) to T lymphocytes. Recently, it has become clear that the breadth of peptide presentation by MHC class I molecules is inversely correlated with the level of cell surface expression, a relationship that is correlated with resistance to Marek's disease in chickens and with progression to AIDS in humans. In this article, evidence is presented that class I molecules vary in a suite of correlated properties, including thermostability, that are influenced, at least in part, by the breadth of peptide translocation by the transporters for antigen presentation (TAPs) that pump peptides to be loaded.

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Fig. 1. High-expressing haplotypes produce class I molecules that are more thermostable than low-expressing haplotypes. Detergent lysates of chicken erythrocytes or blood PBLs were incubated at the indicated temperatures, IPs with the mAb to β_2m were analyzed by SDS gel electrophoresis, followed by WB using the mAb to HC, and the amount of HC was quantified by densitometry after fluorography (representative experiment in Fig. S1). Results from four experiments were normalized and averaged, with SEM indicated by error bars.

might be a difference in overall stability of class I molecules from normal cells, so we examined normal cells with a thermostability assay (Fig. 1 and Fig. S1). Aliquots of detergent lysates from erythrocytes or peripheral blood lymphocytes (PBLs) were incubated at a range of temperatures, followed by immunoprecipitation (IP) with a monoclonal antibody (mAb) against β_2 -microglobulin (β_2m) and then Western blot (WB) with a mAb to the class I heavy chain (HC). Dissociation occurred at lower temperatures for class I molecules from low-expressing haplotypes than high-expressing haplotypes, from a mean of less than 40 °C for B21 to more than 50 °C for B19, although the exact values varied slightly in the five repeats. It is not clear whether this property reflects the kind of peptides bound, some intrinsic property of the HC, or both.

High- and Low-Expressing Haplotypes Produce the Same Amount of Class I Protein Within the Cell, but Transport Different Amounts to the Cell Surface. In fact, the differences in cell surface levels of chicken class I molecules between MHC haplotypes could be a result of differences at many steps in synthesis and turnover. We examined these steps by pulse/chase experiments, with Con A-stimulated PBLs pulsed with radioactive Met and chased with excess unlabeled Met, and with IP of fully assembled and stable class I molecules by the mAb against β_2m before analysis by SDS/PAGE. Many preliminary experiments established that roughly the same amount of radioactive Met was incorporated into HC associated with β_2m in all haplotypes (e.g., Fig. S2).

To distinguish between class I molecules on the inside of the cell and those on the cell surface, two-stage IPs were carried out (e.g., Fig. 2). First, live intact cells on ice were coated with the mAb, washed thoroughly, and lysed with detergent (with care taken to exclude dead cells and to mop up free mAb binding sites on lysis) before the first IP step, which gave molecules on the outside of the cell. After clearing any mAb that might have escaped the first IP step, a second IP with the mAb to β_2m was carried out on the resultant lysate, which gave molecules from the inside of the cell.

The results of densitometry in this experiment show that roughly the same amount of radioactivity was incorporated into class I molecules containing β_2m in both B15 and B21 cells, with virtually all of the radioactivity still in the inside of the cell at the start of the chase ($t = 0$ h). By 1.5 h of chase (the maximum surface expression for such cells under these conditions, as determined by preliminary experiments), 35% of the class I molecules were on the surface of B15 cells compared with 13% for B21 cells (similar to PhosphorImager analysis, 33% vs. 15%; raw data in the legend to Fig. 2), which compares well with the relative fluorescence of these same cells by flow cytometry. These

analyses showed that the difference was accounted for by molecules remaining inside the cell (see legend to Fig. 2). By 4.5 h of chase, the labeled molecules are nearly undetectable under these conditions. Similar experiments showed that Con A-stimulated PBLs from several high- and low-expressing haplotypes synthesize the roughly same number of class I molecules, but differ in the amount that reaches the cell surface. The pulse/chase shows that the class I molecules from all haplotypes examined have no large differences in the rate of translocation to the surface or the rate of degradation.

Given the results of the thermostability experiment, it was possible that the same number of class I molecules reached the surface of cells, but more class I molecules dissociated at the cell surface of low-expressing haplotypes. This seems unlikely, given that more labeled class I molecules are found inside B21 cells compared with B15 cells at 1.5 h in the pulse/chase experiment (Fig. 2). To examine this possibility in another way, PBLs of several MHC haplotypes were incubated overnight with high-affinity synthetic peptides (based on those isolated from the surface of cells), and then cell surface expression was examined by flow cytometry (Fig. S3) in an assay analogous to the class I stabilization assays often used for mammalian cells lacking TAP activity (10, 19). For every haplotype tested, the relative level of class I molecules increased (~6–48%), but the low-expressing haplotype B21 was not rescued to a greater extent (~32%) than the high-expressing haplotypes. As a third way to examine this point, cells were incubated overnight at varying temperatures (Fig. S4), similar to experiments using mammalian cells lacking TAP activity (20). There are slight differences in class I expression on the cell surface at different temperatures, but the low-expressing B21 haplotype did not increase to the level of the other haplotypes. These experiments show that there is not obviously an excess of empty or very unstable class I molecules on the surface of B21 cells, and that transport to the cell surface is the major factor between high and low expression.

Class I Molecules from High-Expressing Haplotypes Can Bind a Much Wider Variety of Peptides *In Vitro* than Are Found on the Cell Surface. The acquisition of peptides in the PLC is a major determinant for transport to the cell surface. We have found that the peptide

Fig. 2. High- and low-expressing haplotypes produce the same amount of class I protein within the cell, but transport different amounts to the cell surface. Con A-stimulated PBLs from line H.B15 (Left) and H.B21b (Right) chickens were labeled with radioactive Met for 30 min, chased for the indicated times, incubated with mAb to β_2m and lysed with detergent, and the mAb-bound cell surface class I molecules were precipitated with protein A-beads (outside). The supernatant of the IP was incubated with mAb to β_2m and the mAb-bound class I molecules precipitated with protein A-beads (inside). All IPs were analyzed by SDS gel electrophoresis and fluorography (S, standard proteins at 20, 30, 45, 70, and 95 kDa). The intensity of the HC band at 1.5 h was 64.54 by densitometry (10,587 by PhosphorImager) for inside and 37.67 (5,324) for outside of B15 cells, and 82.08 (16,357) for inside and 12.38 (2,791) for outside of B21 cells. Flow cytometric analysis showed mean fluorescence intensity of 775 (F21-2) and 1,942 (F21-21) for B15 blasts and 197 (F21-2) and 830 (F21-21) for B21 blasts.

motif of fastidious class I molecules in cells matches the translocation specificity of the linked TAP alleles (16). Nearly all single peptides eluted from B15 chicken cells have the same anchor residues (10), an Arg or Lys at peptide position P1, an Arg at P2, and a Tyr at Pc (with trace amounts of Phe and Trp found at Pc by pool sequencing). However, we had found that some peptides with Gly or Thr at P1, His at P2, and Val or Leu at Pc led to increases in cell surface class I levels after overnight incubation with PBLs followed by flow cytometry (Fig. S5).

To examine this issue more carefully, three peptide libraries based on the 8mer KRLIGRKY were synthesized, each library with one position (P1, P2, or Pc) having 19 amino acids (all but Cys) at roughly equal proportions. Each library was assembled with β_2m and the BF2*1501 HC, the components were separated by size-exclusion chromatography, and the class I monomer peak was analyzed by reverse-phase HPLC to separate the peptides (Fig. 3).

The results show that many peptides from each library failed to assemble with BF2*1501. However, instead of just the basic amino acids Arg and Lys at P1, peptides with the hydrophobic amino acids Tyr, Leu, Ile, Val, and Ala (and/or Thr) also assembled with class I molecules. Instead of just the basic Arg at P2, both Arg and Lys were found, and instead of just Tyr (with a little Phe and Trp) at Pc, Leu and Ile as well as basic Arg, Lys, and His were found. Similar results were found after analysis by mass spectrometry, with or without incubation at 32 °C (Fig. S6). Moreover, such results were recently reported for BF2*0401, in which various amino acids at anchor residue positions allowed assembly of synthetic peptides (21). Thus, it appears that class I molecules from high-expressing haplotypes can bind a wider variety of peptides than are found on the surface of cells.

TAPs Vary Enormously in the Variety of Peptides Transported. On the basis of the results of TAP transport assays (16) and the data

presented here, it appears that the TAP translocation specificity can restrict the peptides found on the cell surface of high-expressing haplotypes. The class I molecules from low-expressing haplotypes can bind an astonishing variety of peptides (11, 14), but the specificity of such TAPs have never been reported. We conducted translocation inhibition assays with a variety of peptides, using permeabilized B15 and B21 cells, comparing the extent of TAP transport with class I binding (Fig. 4).

The B15 and B21 peptides used for transport are based on natural peptides eluted from cells, single amino acid swaps, and peptides from pathogens predicted based on anchor residues. Several peptides found at the surface of B15 cells were not transported well by B15 cells, whereas peptides were transported by B21 cells that did not assemble well with the class I molecule BF2*2101 (Fig. 4). Thus, B15 TAPs are restrictive, in that they limit the peptides presented by the class I molecule on B15 cells, whereas B21 TAPs are permissive, in that they pump a wider variety of peptides that more than match the promiscuous binding of the dominantly expressed class I molecules on B21 cells.

B15 Class I Molecules from B15/B21 Heterozygote Animals Are Expressed at a Higher Level than Those from B15 Homozygotes.

Given that TAPs restrict the peptides received by high-expressing class I molecules, it seemed possible that class I molecules from one MHC haplotype might receive additional peptides from the TAP of another haplotype (16). We examined the expression level of class I molecules on PBLs from the progeny of matings between B15 and B21 chickens (one F2 family and two F1 families), all with similar results (e.g., Fig. 5). The mAb to β_2m stained B21/B21 cells less than B15/B15 cells, whereas a mAb to B21 class I molecules stained the B21/B21 cells and not the B15 cells. One might have expected staining by the mAb to β_2m to be intermediate in B15/B21 cells, but in fact it was greater than B15/B15 homozygotes. The mAb to B21 molecules stained heterozygote cells roughly half as much as the B21/B21 homozygote cells. Overall, it seems likely that the cell surface expression of B15 class I molecules in B15/B21 heterozygotes increases beyond the levels of B15/B15 homozygotes, although from these experiments, we cannot rule out that the minor B21 molecule BF1*2101 or another unidentified class I molecule has increased in amount. These data are similar to results of radioimmunoassays using alloantisera for B15 and B21 class I molecules reported long ago (22).

Discussion

We have reported that the cell surface expression of chicken class I molecules varies inversely with diversity of peptide repertoire and with resistance to Marek's disease, and that such correlations are found for some human class I molecules (6, 10, 11, 14). Here we make three major points about these findings in chickens: that the control of cell surface expression of class I molecules is determined by some aspect of translocation to the surface, that TAP specificity controls the peptides bound by and the cell surface expression of fastidious class I molecules, and that both peptide translocation of TAPs and thermal stability of class I molecules are part of this suite of correlated properties.

The level of chicken class I molecules on the surface of cells appears to be determined by the number of molecules that move to the surface, and not by transcription, translation, kinetics of translocation, or kinetics of degradation, as assessed by pulse/chase experiments. Moreover, adding high-affinity peptides and culturing the cells at lower temperatures both suggest that the cell surface expression is not a result of differential degradation of class I molecules that bear very low affinity (or no) peptides. These data are consistent with the fact that the dominantly expressed class I genes all have nearly identical promoters and 3' UTRs (18) and point to the importance of the PLC, which in chickens has polymorphic TAPs and tapasin that affect function

Fig. 3. The B15 class I molecule binds peptides with residues in anchor positions beyond those found in the peptide motif determined from B15 class I molecules on the surface of cells. HPLC reverse-phase chromatography of peptide libraries based on the B15 peptide KRLIGRKY with 19 amino acids in position 1 (A and B), 2 (C and D), and 8 (E and F), either without treatment (A, C, and E) or after assembly with and elution from the B15 class I molecule (B, D, and F), with residues in assembled peptides indicated by a single-letter code.

Fig. 4. B15 TAP transports a very restricted group of peptides, whereas B21 TAP transports a wider variety of peptides than is bound by the class I molecule BF2*2101. For the transport assay, permeabilized TG15 (Left) and TG21 cells (Right) were incubated with radiolabeled synthetic peptides P198 (KRYNASAY) and p536 (TNPSSKVFYL), respectively, and then lysed with detergent, and the amount of radioactivity bound to Con A-beads taken to indicate peptide translocation followed by glycosylation. The amount of radioactive peptide used was set at 50% maximal transport, and nonradioactive synthetic peptides (at concentrations equivalent to the amount of transport peptide needed for 50% inhibition) were added to assess inhibition (taken to indicate binding and/or transport), with error bars indicating SEM. Peptides (including duplicate syntheses) based on those eluted from B15 and B21 cells are marked with asterisks. Assembly assays and cell stabilization experiments (performed 1–5 times each) were used to assess the binding of synthetic peptides. For cell stabilization experiments, significant binding is indicated by green bars, marginal binding by orange bars, and no binding by red bars. For the assembly assays, stable binding is indicated by green bars, unstable binding by orange bars, and no binding by red bars. Unstable binding included broad monomer peaks in the FPLC trace as well as inconsistent results in different experiments; no binding included no peak or a peak at the position of HC refolded alone. Experiments not done are indicated by open bars.

(15–17). However, the same phenomena may occur in mammals, which have effectively monomorphic TAPs and tapasin. The inverse correlation between cell surface expression level and peptide repertoire is found for at least some HLA-B alleles in humans (14). Long ago, differences of human class I alleles in transport to the cell surface were reported (23). Differences in assembly of HLA-B alleles are reported to be a result of polymorphic HLA-B residues involved in interaction with tapasin (24), with the rank order of the four HLA-B alleles matching that reported for cell surface expression level and peptide repertoire (14).

In chickens, polymorphic class I, TAP, and tapasin molecules are present in the PLC, so the variation in any of these molecules may contribute to the expression level at the cell surface. We have previously shown that the structural polymorphism of the TAPs can lead to functional differences in translocation specificity (16). Here we report that the TAP from the fastidious B15

haplotype restricts the peptides available to the class I molecules, with the fastidious BF2*1501 class I molecule actually able to bind a much wider variety of peptides than those found on the surface of B15 cells. Similar data were also reported for the B4 haplotype (21). Such a phenomenon has recently been reported for a mouse class I molecule (25), with H-2K^b initially binding a wide range of anchor residues that are then self-edited in a temperature-dependent step. We find no enormous temperature-dependent effect with the chicken molecule BF2*1501, consistent with the restriction of peptides by TAP translocation. However, we also find that the TAP from the promiscuous B21 haplotype has much wider translocation specificity, pumping peptides that are not bound well by the dominantly expressed BF2*2101 molecule, indicating there is a range of specificities of TAP transport in chickens that correlates with the properties of the dominantly expressed class I molecule. One interesting

Fig. 5. B15 class I molecules from B15/B21 heterozygote animals are expressed at a higher level than from B15 homozygotes. PBLs from the progeny of two F1 crosses of B15 and B21 chickens (F, female parent; M, male parent) were assessed by flow cytometry using the mAb F21-21 to β_2m and the mAb 37C.18 to B21 (but not B15) class I molecules. Parents and nine progeny at 9 months of age were analyzed, with error bars indicating SEM for each set of progeny.

consequence might be that the class I molecules in chicken MHC heterozygotes could receive peptides not available in homozygotes. In fact, we find that the expression level of class I molecules in B15/B21 heterozygotes is increased compared with B15/B15 homozygotes, similar to a study buried in the scientific literature (22). As previously suggested (16, 26), TAP polymorphism may increase the peptides available in heterozygotes compared with homozygotes, and thus can add another molecular layer to selection by heterozygote advantage (overdominance) determined by the chicken MHC.

Our previous work suggested two groups (or a hierarchy between two extremes) of class I molecules with different strategies for disease resistance, based on the inverse correlation of expression level and peptide repertoire (14). Here we show that in chickens, there is a suite of correlated properties, including TAP specificity (as just discussed) and thermal stability of class I molecules. The promiscuous class I molecules are slightly less stable than the fastidious molecules, in a rank order similar to that of the expression levels, but it is not clear whether this is an intrinsic property of the class I molecule (how HC folds up, affinity for β_2m , etc.) or a property of the peptides bound. It is somewhat surprising that this difference in stability has no obvious effect on the kinetics of degradation, an issue that requires further examination. Also, it is not clear whether this difference in stability will be found for class I molecules in typical mammals. Examination of the human class I alleles with different peptide repertoires and/or expression levels (12–14) is one approach. More dramatic differences might be found with class I molecules from chimpanzees, which include two groups (27, 28): one group with fastidious peptide motifs very much like the fastidious human HLA-B*5701 and B*2705 molecules and another group more like the promiscuous chicken BF2*0201 and BF2*1401 molecules.

Materials and Methods

Animals and Cells. All experiments involving animals were carried out with appropriate review by the institutions under the laws of the country at the time. Experimental lines of chickens with known MHC haplotypes were kept in Basel (Figs. 2 and 5 and Figs. S2–S5), Compton, and Cambridge (Figs. 1 and 4 and Fig. S1), as described (10, 18, 29, 30). F1 families based on H.B15 and H.B21b chickens led to B15/B21 progeny at the Gipf (Oberfrick) Farm of the Basel Institute for Immunology (Fig. 5). Previously described are isolation of erythrocytes and PBL, using slow-speed spin and Ficoll gradient centrifugation, culture of blood PBLs in DMEM with 10% (vol/vol) FBS, 1% selected chicken serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin (Fig. 2 and Figs. S2 and S4) maintained at 37 °C or 40 °C with 5% (vol/vol) CO₂ (10, 31). Also described (10, 18) is culture of ex vivo PBLs either with stimulation by 5 μ g/mL Con A (Sigma) for 3 d in culture (Fig. 2 and Figs. S2 and S4), or with and

without 1 mM synthetic peptides in DMEM with 0.5 mg/mL BSA overnight (Fig. 4 and Fig. S3). The REV-transformed cell lines TG15 and TG21 (Fig. 4) are described (16).

Antibodies, IP, WB, and Flow Cytometry. The mAb F21-2 directed to all chicken HC, F21-21 to chicken β_2m , and 37C.18 that binds class I molecules from B21 but not B15 cells have been described (32, 33). Previously described are general procedures for IP, electrophoresis using Laemmli SDS 12% polyacrylamide gels and fluorography (Fig. 2 and Fig. S2, ref. 34; Fig. S1, ref. 16, but with modifications noted here), WB procedures by semidry blotting (16), and flow cytometry procedures (Figs. 2 and 5 and Figs. S3–S5; ref. 35).

Thermostability Assays (Fig. 1 and Fig. S1). Based conceptually on ref. 36, erythrocytes were lysed on ice at 10⁸/mL in 2% (vol/vol) Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl at pH 8, 1 mM MgCl₂, 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Pefabloc, Sigma), and cleared of subcellular debris by centrifugation for 10 min at 17,000 $\times$ g at 4 °C in Fesco17 centrifuge. Aliquots of cleared lysate (100 μ L for B21, B2, and B14; 25 μ L for B12, B15, and B19) were incubated for 30 min at various temperatures, cooled on ice, and spun again as earlier. The supernatants were used for IP with F21-21 and protein G-beads (with washing by 0.1% Nonidet P-40, 50 mM Tris-Cl at pH 8, 150 mM NaCl), followed by SDS gel electrophoresis with MagicMark XP Western Standards (Invitrogen) and WB with F21-2 and HRP-conjugated anti-mouse IgG Fc-specific (Sigma).

Pulse-Chase Experiment (Fig. 2). Con A-stimulated PBLs (1.5 $\times$ 10⁸ cells each) were treated with 1.25% (mass/vol) α -methyl mannoside for 30 min at 37 °C and washed with warm Met-free medium (Selectamine kit; GIBCO) with 1.5% (vol/vol) FBS (previously dialyzed against PBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin, resuspended in the same medium (5 mL) with 2.75 mCi (101.75 MBq) ³⁵S-Met and incubated for 30 min at 37 °C to pulse-label. The chase was begun by the addition of 5 mL medium with 0.071 mg/mL nonradioactive Met, and at each point, 2.5 mL were removed, with all subsequent steps at 4 °C or on ice, with cold buffers. The cells were centrifuged immediately at 1,000 rpm for 6 min in a Heraeus centrifuge, resuspended in 2 mL PBS with 0.5 mg/mL BSA, 0.1% NaN₃ (PBS/BSA/Az), underlaid with Ficol-paque, and centrifuged as earlier. The interface containing live cells was collected, washed with 12 mL PBS/BSA/Az buffer as earlier, resuspended in 0.1 mL PBS/BSA/Az containing 5 μ L F21-21 ascites, incubated for 30 min, and washed three times as earlier with a change of tubes. The cell pellet was lysed in 0.5 mL 2% (vol/vol) Nonidet P-40, 100 mM Tris-Cl at pH 8, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM phenyl methyl sulfonyl fluoride (in which chicken erythrocytes had previously been lysed, to provide unlabeled class I molecules to block any free antibody binding sites) for 15 min. The lysate was transferred to a 1.5-mL microfuge tube and centrifuged at 13,000 rpm for 5 min in an Eppendorf centrifuge. The supernatant was transferred to another tube containing 20 μ L 50% (vol/vol) protein A-beads in PBS/BSA/Az, incubated for 30 min with occasional inversion, and centrifuged at 1,000 rpm for 2 min to give the cell surface class I molecules (“outside”). To “preclear” the supernatant after the protein A-bead precipitation (and sop up any antibody that had not been removed), 5 μ L normal rabbit serum was added and incubated for 1 h before addition of 40 μ L 50% (vol/vol) protein A-beads and incubation with rotation for 40 min, followed by centrifugation at 13,000 rpm for 5 min. The supernatant was transferred to another tube with 5 μ L F21-21 and incubated for 1 h, before addition of 20 μ L 50% (vol/vol) protein A-beads and incubation with rotation for 40 min, followed by centrifugation at 1,500 rpm for 2 min (“inside”). The IP were washed with NET buffers, boiled in sample buffer with 5% (vol/vol) 2-mercaptoethanol, resolved by SDS gel electrophoresis and detected by fluorography after soaking the gel in 0.5 M sodium salicylate, as described (10, 34).

Assembly of Denatured Class I Heavy Chains and β_2m with Peptides and Peptide Libraries (Figs. 3 and 4). Overall, methods are described, including bacterial expression and purification of protein chains (11). Small-scale assembly assays were carried out at 4 °C with vigorous stirring in 1 mL refold buffer (100 mM Tris-Cl at pH 8.2, 400 mM arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, 2 mM EDTA, 0.1 mM AEBSF). The peptide, β_2m , and heavy chain were added slowly with vigorous stirring in a molar ratio of 10:2:1 (recently roughly 2.5 μ g:6 μ g:7 μ g). After 18–42 h, proteins and smaller molecules were resolved by FPLC size-exclusion chromatography (AKTA; Pharmacia), using HiLoad 26/60 Superdex 75, Superdex 75 HR 10/30, Superdex 200 HR 10/30, or Superdex 200 10/300 GL columns (Pharmacia), most recently with 25 mM Tris-Cl at pH 8, 100 mM NaCl, 0.1% NaN₃.

Assembly with B15 peptide libraries was carried out as earlier, but scaled to 50 mL refold buffer stirred slowly at 4 °C, with first 416 μ g peptide library

(xRLIGRKY, P139; KxLIGRKY, P140; KRLIGRKY, P141; made by fMOC chemistry at the IAH Protein Chemistry Facility) and then 1.33 mg β_2m added by pipette, and then 1.6 mg B15 HC added slowly by dripping at $1 \times$ gravity through a 23-g needle. The solution was stirred slowly at 4 °C for 2 d and then clarified by centrifugation at 4,000 rpm for 10 min at 4 °C, using a Sorvall RC-3B centrifuge. The supernatant was concentrated to 6 mL by centrifugation in 15-mL spin concentrators (Ultrafree, Amicon) at 2,000 rpm at 4 °C, using a Sorvall RC3B. The monomers were isolated by FPLC size-exclusion chromatography (AKTA; Pharmacia), using on a Superdex SD75 column (Amersham) with 100 mM Tris-Cl at pH 8, 150 mM NaCl. The fractions containing monomers were concentrated to $\sim 100 \mu\text{L}$, using Ultrafree 15 mL and then Centricon 10 spin concentrators. The peptides were eluted by 0.1% trifluoroacetic acid, isolated using the Centricon 10 spin concentrator, and concentrated by use of a speed-vac. Both the original peptide library and the eluted peptides were separated by reverse-phase HPLC, using a C18 Sephasil 5 μm SC 2.1/10 column on a SMART system (Pharmacia Biotech) with a gradient of 0–40% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid.

Peptide Translocation Assays (Fig. 4). TG15 and TG21 cells were permeabilized and incubated with iodinated synthetic indicator peptides in the presence or absence of unlabeled synthetic inhibitor peptides (0.15 μM for B15 and 0.1 μM for B21, based on 50% inhibition by the appropriate indicator peptide) for 5 min at 37 °C and lysed with detergent, essentially as described (16), but modified for higher throughput. The reaction volume was halved, but with the same number of cells. After cell lysis and centrifugation, the

lysate was incubated with occasional inversion at 4 °C overnight with Con A Sepharose (Sigma) in empty size-exclusion columns (Epoch Life Science, cat. no. 2070). Supernatant containing unbound labeled peptide was removed, and the beads were washed three times with 0.1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl at pH 8 by gravity flow. The size-exclusion columns including beads were analyzed using a 1470 Wizard gamma counter (Wallac). Preliminary experiments showed that TG15 cells transported little P258 (KMYNASAY), and that transport into TG21 cells by p300 (GHAENYSAETL) was more strongly inhibited by many tested peptides than it was by itself, but P198 (KRYNASAY) and p536 (TNPSSKVFYL) had high transport rates and were among the highest inhibitors of transport for TG15 and TG21, respectively.

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