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 ten-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 shows 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 due to 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 | heterozogous advantage | overdominance

Introduction

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 (NK) 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 non-progression to acquired immunodeficiency syndrome (AIDS) in humans (12, 14). Based on 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). Co-evolution 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 the 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

Significance

Major histocompatibility complex (MHC) molecules play crucial roles in the immune response to pathogens and tumours 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 progression to AIDS in humans. In this paper, 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) which pump peptides to be loaded.

Reserved for Publication Footnotes
High expressing haplotypes produce more thermostable class I molecules than low expressing haplotypes. Detergent lysates of chicken erythrocytes or blood PBLs were incubated at the indicated temperatures, IPs with the mAb to β2m 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.

Con A-stimulated PBLs from line H.B15 (left) and H.B21 (right) chickens were labeled with radioactive Met for 30 min, chased for the indicated times, incubated with mAb to β2m, lysed with detergent and the mAb-bound cell surface class I molecules precipitated with protein A-beads (outside). The supernatant of the IP was incubated with mAb to β2m 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 ± 8.4 by densitometry (10587 by phosphoimager) for inside and 37.67 (5324) for outside of the cell. Chase for 15, 45, 90, 180 and 360 min with Met, and the mAb-bound HC was precipitated on protein A-beads and washed and then analyzed by WB using the mAb to HC, and the amount of HC was quantified by densitometry after 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 ± 8.4 by densitometry (10587 by phosphoimager) for inside and 37.67 (5324) for outside of the cell.

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, b), 2 (c, d) and 8 (e, f), either without treatment (a, c, e) or after assembly with and elution from the B15 class I molecule (b, d, f), with residues in assembled peptides indicated by single letter code.

In order to distinguish between class I molecules on the inside of the cell and those on the cell surface, two-stage IPs were carried out (for example, Fig. 2). First, live intact cells were coated with the mAb on ice, washed thoroughly and lysed with detergent (with care taken to exclude dead cells and to mop up free mAb binding sites upon 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 β2m was carried out on the resultant lysate, which gave molecules from the inside of the cell.
Fig. 4. B15 TAP transports a very restricted group of peptides, while 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 radiolabelled 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 utilized was set at 50% maximal transport, and non-radioactive 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; M. Harrison, A. van Hateren and J. Kaufman, unpublished data) 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.

The results of densitometry in this experiment show that roughly the same amount of radioactivity was incorporated into class I molecules containing β2m 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=0h). 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 to 13% for B21 cells (similar to phosphoimager analysis, 33% versus 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 to 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 employed for mammalian cells lacking TAP activity (10, 19). For every haplotype tested, the relative level of class I molecules increased (~6% to 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), like similar 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 motif of fastidious class I molecules in cells match 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 Pe (with trace amounts of Phe and Trp found at P6 and Pe by pool sequencing). However, we had found that some peptides with Gly or Thr at P1, His at P2, and Val or Leu at Pe led to increased cell surface class I levels after overnight incubation with PBLs followed by flow cytometry (Fig. S5).

In order to examine this issue more carefully, three peptide libraries based on the 8-mer KRLIGRRKY were synthesized, each library with one position (P1, P2 or Pe) having 19 amino acids (all but Cys) at roughly equal proportions. Each library was assembled with β2m 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 Pe, 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.**

Based on the results of TAP transport assays (16) and the data presented above, 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 present a much more 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, while 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, while B21 TAPs are permissive in that they pump a wider variety of peptides that might 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 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 (for example, Fig. 5). The mAb to β2m stained B21/B21 cells less than B15/B15 cells, while a mAb to B15 class I molecules stained the B21/B21 cells and not the B15 cells. One might have expected staining by the mAb to β2m to be intermediate in B15/B21 cells, but in fact it was greater than B15/B15 homoygotes. The mAb to B21 molecules stained heterozygote cells roughly half as much as the B21/B21 homoygote 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 homoygotes, 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 due to 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 have polymorphic TAPs and tapasin that affect function (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 chickens have polymorphic TAPs and tapasin that affect function (overdominance) determined by the chicken MHC.

Another molecular layer to selection by heterozygote advantage (between two extremes) of class I molecules with different strategies (positive and negative selection) are present in the PLC, so the variation in any of these molecules is determined by some aspect of translocation to the surface, the cell surface expression level and peptide repertoire (14). Here we show 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 beta 2 fastidious BF2*1501 class Imolecule 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).

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).

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by SDS gel electrophoresis and detected by fluorography after soaking the gel in 0.5 mM sodium selenite as described (34).

Assembly of denatured class I heavy chains and β2m with peptides and peptide libraries (Fig. 3 and 4). Overall, methods are described, including bacterial expression and purification of protein chains (11). Small-scale assembly was carried out at 4°C with vigorous stirring in 1 ml refold buffer (100 mM TrisCl pH 8.2, 400 mM arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, 2 mM EDTA, 0.1 mM ABEFS). The β2m and heavy chain were added slowly with vigorous stirring in a molar ratio of 10:21 (recently 2.5 μg 6 μg; 7 μg). 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 10/30 or Superdex 200 10/300 GL columns (Pharmacia), most recently with 25 mM TrisCl pH 8, 100 mM NaCl, 0.1% Na2S2O4.

Assembly with B15 peptide libraries was carried out as above, but scaled to 50 ml refold buffer stirred slowly at 4°C, with first 416 μg peptide library (xRLGKY, P139; xLIGRKY, P140; KRLIGKRx, P141; made by fMOC chemistry and Biological Sciences Research Council (BBSRC) of the UK) and finally by programmatic grant 08305 from the Wellcome Trust to JK.

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