Poor assembly of class I major histocompatibility HLA-C heavy chains results in their intracellular accumulation in two forms: free of and associated with their light chain subunit (β2-microglobulin). Both intermediates are retained in the endoplasmic reticulum by promiscuous and HLA-dedicated chaperones and are poorly associated with peptide antigens. In this study, the eight serologically defined HLA-C alleles and the interlocus recombinant HLA-B46 allele (sharing the HLA-C-specific motif KYRV at residues 66–76 of the α1-domain α-helix) were compared with a large series of HLA-B and HLA-A alleles. Pulse-labeling experiments with HLA-C transfectants and HLA homozygous cell lines demonstrated that KYRV alleles accumulate as free heavy chains because of both poor assembly and post-assembly instability. Reactivity with antibodies to mapped linear epitopes, co-immunoprecipitation experiments, and molecular dynamics simulation studies additionally showed that the KYRV motif confers association to the HLA-dedicated chaperones TAP and tapasin as well as reduced plasticity and unfolding in the peptide-binding groove. Finally, in vitro assembly experiments in cell extracts of the T2 and 721.220 mutant cell lines demonstrated that HLA-Cw1 retains the ability to form a peptide-receptive interface despite a lack of TAP and functional tapasin, respectively. In the context of the available literature, these results indicate that a single locus-specific biosynthetic bottleneck renders HLA-C peptide-selective (rather than peptide-unreceptive) and a preferential natural killer cell ligand.

Class I human leukocyte antigens (called HLA) are cell-surface heterotrimers formed by a highly polymorphic heavy (44 kDa) chain, a non-polymorphic light (12 kDa) chain subunit (β2-microglobulin (β2m))3, and a short (8–11-mer) peptide antigen derived from the degradation of intracellular proteins (1). The assembly pathway of most class I molecules involves an early interaction of the heavy chain, still free of β2m, with calnexin, followed by association with β2m and binding to the so-called peptide-loading complex. This is a supramolecular endoplasmic reticulum structure comprising, among others, two HLA-dedicated chaperones: TAP transporter associated with antigen processing (TAP) and the peptide editor/facilitator tapasin (1). Successful peptide loading results in tight association of the heavy chain with β2m and the release of thermally stable, folded class I conformers (1–3). These are exported to the cell surface, where they activate and inhibit cytotoxic T lymphocytes expressing the rearranging T cell receptor and natural killer (NK) cells expressing non-rearranging receptors such as the killer immunoglobulin-like receptors, respectively (4).

There are >1000 class I molecules, encoded by three highly polymorphic allelic series: HLA-A, -B, and -C (www.anthonyonolan.org/UK/HIG/index.html). They share a conserved general architecture, a common peptide-loading pathway, and a similar set of functions, but also display a number of allele- and locus-specific sequences responsible for distinctive structural properties and specialized functional features (reviewed in Ref. 1). In the case of HLA-C, there are three known locus-specific motifs, one of which (particularly extensive) involves heavy chain residues 66, 67, 69, and 76 (KYRV) in the α1-domain α-helix (5). Next to the KYRV 66–76 motif, a functional dimorphism involving residues 77 and 80 regulates NK cell recognition by the KIR2DL1/KIR2DL2 inhibitory receptors (4). Because crystallographic data show that T cell receptor and killer immunoglobulin-like receptor footprints on HLA-C are largely overlapping and that both encompass the HLA-C-specific motif (6), it appears that a stretch of polymorphic residues on the α1-domain α-helix encodes a unique recognition structure promiscuously recognized by HLA-C-specific cytotoxic T lymphocytes and NK cells. Relevant to this tight packaging of distinct structural determinants within a narrow region, it appears that HLA-C, although an extremely good ligand for NK cells, is a much poorer antigen-presenting class I molecule than HLA-A and HLA-B (reviewed in Refs. 1, 5, and 7). Also in contrast to HLA-A and HLA-B, HLA-C is a rather poor assembler. This latter feature has been proposed to correlate with impaired intracellular transport and low surface expression (8, 9), although this conclusion has been questioned (10). Poor assembly of HLA-C is strongly supported by the intracellular accumulation of two broad classes of folding intermediates: β2m-free and β2m-associated class I heavy chains. HLA-C heavy chains free of β2m (free heavy chains) were the first folding intermediates to be identified and extensively char-
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characterized (8) based on their reactivity with antibodies that bind linear epitopes accessible on unfolded but hidden in B2m-associated heavy chains. More recently, taking advantage of two such antibodies (HC10 and L31) to mapped, nearly contiguous linear epitopes spanning the entire N-terminal region of the α1-domain α-helix (L31 binds an epitope centered on an aromatic amino acid at position 67), we have provided evidence that free HLA-C heavy chains bear an extensive local unfolded involving the HLA-C-specific 66–76 motif and crucial peptide-anchoring positions in the so-called B-pocket of the antigen-binding groove (11). We have also shown that free HLA-C heavy chains are thermally unstable, peptide-free, and essentially unreactive to peptides in vitro assembly assays, i.e. their binding groove is in an “open” (as defined in Refs. 2 and 3) conformation. Consistent with these biochemical features, the latter are defective in HLA-A and HLA-B, but retain ~50% of HLA-Cw*0102 (Cw1) expression and, in addition, lack functional tapasin (16). The TAP-defective 174×CEM.T2 cells (referred to as T2) express HLA-A2, -B51, and -Cw1 (19) heavy chains in the absence of TAP as a result of somatic hybridization between the TAP-defective 174 cell line (also a derivative of 221) and the T lymphoid cell line CEM. The HLA-A, -B, and -C transfectants in 221 were obtained through the courtesy of several investigators (see “Acknowledgments”) and are referenced elsewhere (14). Epstein-Barr virus-transformed, HLA-typed, and homozygous B cells are also described elsewhere (12, 14).

Antibodies—The murine monoclonal antibodies HC10 and L31 bind linear α1-domain epitopes including residues 62 (20) and 67 (14), respectively. Q1/28 binds an α3-domain epitope (21). F4/326 (22) and W6/32 (23) bind class I major histocompatibility complex heavy chains associated with B2m. Rabbit polyclonal antibodies to TAP1 and tapasin have been described previously (24). Polyclonal antibodies to calnexin and calreticulin were from StressGen Biotechnologies (Victoria, Canada).

Immunochromical Methods—Cells were metabolically labeled with [35S]methionine (9.25 MBq/ml) as described in the figure legends and solubilized with either 1.0% Nonidet P-40 or 0.5% CHAPS in phosphate-buffered saline (0.01 M phosphate (pH 7.0) and 0.15 M NaCl). For immunoprecipitation, purified antibodies were covalently linked to Affi-Gel (Bio-Rad). The isoelectric focusing (IEF) and Western blot techniques (reducing conditions in all cases) used have been described (11, 12, 14). In vitro assembly experiments were performed exactly as described in the presence of the NCPERIITL Cw1 ligand (11).

Molecular Dynamics Simulations—Models of the isolated heavy chains of HLA-B15 (B*1501), HLA-B27 (B*2705), HLA-B46 (B*4601), and HLA-Cw1 (Cw*0102) were created, and molecular dynamics simulations were performed and analyzed using InsightII, Biopolymer, Discover3, and Analysis (Accelrys, San Diego, CA). No explicit water molecules were included, and electrostatic forces were modeled using a distance-dependent dielectric constant (4r). The combined valence force field was used with cell multipole summation, fourth order Taylor series expansions, and 1.0-Å update width. Initially, heterotrimer models were created either directly from or by homology with appropriate published crystallographically resolved structures in the Macromolecular Structure Database: HLA-B15 and HLA-B46 from HLA-B*1501 (Protein Data Bank code 1XR9) (25), HLA-B27 from HLA-B*2705 (code 1GE) (26), and HLA-
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An Abundant Pool of Free HLA-C Heavy Chain Conformers in B Lymphoid Cells—It was shown in a previous study (11) that the \( \beta_2 \) subunit-free heavy chain conformers reactive with antibody L31 are unable to stably assemble with peptides and \( \beta_2 \) subunits, whereas the \( \beta_2 \) subunit-associated conformers reactive with F4/326 and W6/32 do assemble with peptides in vivo as well as in vitro. The combined pulse-chase/thermal stability experiment shown in Fig. 1A was carried out in stable Cw1 transfectants of the 221 cell line (221.Cw1) to estimate the turnover of the two conformers and the kinetics of acquisition of thermal stability by \( \beta_2 \) subunit-associated conformers. During the first part of the chase (until the 105-min point), L31 conformers slowly and progressively declined, whereas F4/326 conformers gradually increased (lanes 1–5 and 7–11, respectively), as expected (11, 14), although thermal stability (indicative of stable peptide binding) was acquired not earlier than 45 min from synthesis (compare lanes 7–9 and 13–15 and lanes 10 and 16). From this point on, accumulation of F4/326 conformers and acquisition of thermal stability simultaneously increased, peaked at 105 min (lanes 11 and 17), and then simultaneously declined (lanes 12 and 18).

Based on these kinetics and turnover profiles, a 2-h continuous metabolic labeling can be predicted to provide a cumulative estimate of the stoichiometry of \( \beta_2 \) subunit-free and \( \beta_2 \) subunit-associated conformers throughout the crucial period of productive class \( \alpha \) assembly, before the beginning of disposal. Conformer ratios (a measure of stable folding/assembly) were therefore evaluated under these metabolic labeling conditions in a panel of 221 cells transfected with single class I alleles, including seven serologically defined HLA-C alleles, two HLA-B alleles (HLA-B7 and HLA-B51) representative of good and poor assembly efficiencies, respectively (15), two alleles (HLA-A2 and HLA-B15) lacking the crucial residues forming the optimal L31 epitope (14), and the interlocus recombinant HLA-B46 allele. Representative results of immunoprecipitation experiments with L31 and W6/32 (F4/326 gave similar results; data not shown) are depicted in Fig. 1B.

Whereas W6/32 conformers were detected at high levels in all the 221 transfecteds (Fig. 1B, lanes 2, 5, 8, 11, 14, 19, and 23), L31 conformers ranged from barely detectable in 221.B7 (lane 10) to intermediate levels in 221.B51 (lane 7) and up to levels comparable with those of the W6/32 conformers in 221.Cw1 (lane 1), 221.Cw7 (lane 4), 221.Cw3, 221.Cw4, 221.Cw5, 221.Cw6, and 221.Cw8 (data not shown and see below) (12, 14), and, most notably, 221.B46 (lane 22). HLA-B46 (B*4601) carries the HLA-C-distinctive KYRV motif, absent in HLA-B15 (B*1501), at residues 66–76 in the \( \alpha_1 \)-domain \( \alpha \)-helix (13), but the two alleles are otherwise identical. Consequently, HLA-B15 does not contain residues 66–68 specifying the L31 epitope (also see lane 18) (14). In view of this, a comparison of free heavy chain accumulation between HLA-B46 and HLA-B15 was carried out using Q1/28, an antibody to conserved \( \alpha_3 \)-domain residues preferentially carried by free heavy chains (21). Q1/28 revealed a much greater accumulation of HLA-B46 than HLA-B15 free heavy chains (compare lanes 17 and 21). Thus, HLA-B46 free heavy chains bear at least two local unfolding regions: in the \( \alpha_1 \)-domain \( \alpha \)-helix (e.g. the very site of gene conversion, revealed by L31) and in the \( \alpha_3 \)-domain (revealed by Q1/28). It can be concluded from Fig. 1B that the HLA-C-specific 66–76 motif correlates with and confers poor assembly and folding impairment.

Impaired and Unstable Assembly Is a Locus-specific Feature of HLA-C—The data in Fig. 1 provide initial evidence that the accumulation of L31 conformers is a feature of HLA-C shared with HLA-B46. It remains to be established whether this accumulation results from (a) poor assembly, (b) increased dissociation, or (c) both. In addition, trivial causes of inaccuracy must be excluded, such as (d) the inclusion of non-classical class I HLA molecules (expressed by 221 cells) in the W6/32 conformer pool and (e) different integrated copy numbers of the transfected class I alleles in distinct 221 transfectants. Different copy numbers may result in different levels of class I heavy chain expression against a background of similar (and possibly limiting) class I chaperoning.

To distinguish among features a, b, and c and to rule out features d and e, we determined the extent as well as the kinetics of the accumulation of the different heavy chain conformers by pulse-chase experiments and resolved the individual class I alleles coexpressed in different cell lines by IEF. In addition to 221 transfectants, HLA homozygous B lymphoid heavy chains (17 alleles altogether), three of these (JY, CJO, and EDR) simultaneously express one L31-reactive HLA-B allele and one L31-reactive HLA-C allele, making them suitable for stringent allele
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comparisons. Representative results in two cell lines are shown in Fig. 2, and a synopsis of the densitometric data of HLA-C- and HLA-B-specific bands for all the tested Y/F677+ HLA-C and HLA-B alleles is shown in Fig. 3.

Consistent with feature a (poor HLA-C assembly), all the eight tested HLA-C alleles and the poor assembler HLA-B51 (15) reacted more intensely with L31 than with W6/32 at either or both

![Image 69x471 to 153x611]

FIGURE 2. Pulse-chase analysis of L31-reactive HLA-C and HLA-B alleles. The JY and WT46 cell lines were pulsed with [35S]methionine (5 min), chased for the indicated times (minutes), solubilized with Nonidet P-40, and immunoprecipitated with L31 and W6/32. The IEF banding patterns of HLA-A and HLA-B (brackets) and HLA-C (arrowheads) alleles are indicated. The uppermost form of HLA-Cw7 migrated right above the most basic form of HLA-B7 in lanes 1, 5, and 6.

![Image 185x471 to 281x610]

FIGURE 3. Densitometry of pulse-chase analysis. The same pulse-chase and immunoprecipitation experiments performed in the representative cell lines displayed in Fig. 2 were carried out in HLA homozygous cell lines simultaneously expressing two L31-reactive alleles, e.g., JY (B7 and Cw7), CJO (B35 and Cw4), and EDR (B56 and Cw2), as well as HLA homozygous cell lines and single-allele 221 transfectants expressing one L31-reactive allele, e.g., JHAF (B51), WT46 (Cw5), LBF (Cw6), 221.B*0702, 221.B*4601, 221.B*5101, 221.Cw*0102, 221.Cw*0301, and 221.Cw*0802. Class I heavy chain components were assigned to specific alleles based on previous IEF comparisons with HLA-C transfectants (12, 14). IEF bands were scanned, and densitometric values of all the bands of a given allele in a given immunoprecipitate and at a given time point were summed. This resulted in 104 cumulative estimates of free and β2m-associated heavy chains accumulating over time, sorted by allele (13 panels, one for each L31-reactive allele) and antibody (L31 and W6/32 (C)).
The KYRV 66–76 Motif in the α1-Domain α-Helix Is Involved in Strong TAP/Tapasin Association—Neisig et al. (9) have shown that HLA-C molecules accumulate, free of peptide, in association with TAP. We therefore tested whether the KYRV motif is involved in determining this feature. To this end, we used antibodies to TAP1 to compare the amounts of co-immunoprecipitated heavy chains in CHAPS extracts of 221.Cw1, 221.B46, and 221.B15 transfectants (Fig. 5). In parallel, co-immunoprecipitation was also carried out with antibodies to tapasin. To detect heavy chains, we used three distinct antibodies, including HC10 and Q1/28, which bind conserved α1- and α3-domain epitopes, respectively. HLA-Cw1 and HLA-B46 (but not HLA-B15) heavy chains were detectably associated with TAP as well as tapasin (compare lanes 2/3 with lanes 7/8 and 12/13 and lanes 32/33 with lanes 37/38 and 42/43; also see lanes 22/23 and 27/28). Thus, the KYRV motif promotes the accumulation of HLA-C heavy chains in association with the two HLA-dedicated chaperones of the peptide-loading complex.

In Vitro Assembly of HLA-Cw1 Heavy Chains in Cells Defective in Tapasin or TAP—Although they are unable to become stably assembled and peptide-filled in cells lacking TAP (T2) or functional tapasin (220), HLA-A and HLA-B alleles do retain peptide receptivity, i.e. the ability to assemble in vitro upon incubation with exogenously added peptides (16, 29–35). Because of its peptide selectivity (9), one may hypothesize that HLA-C is more crucially dependent than HLA-A and HLA-B on TAP and tapasin. Because the available information is limited (36), we evaluated the peptide receptivity of HLA-Cw1, an allele that displays a particularly deep folding impairment (Fig. 3) and a rigid binding groove (Fig. 4). In vitro assembly experiments were performed on soluble extracts of the partially isogenic (see “Experimental Procedures”) T2 and 220 cells, both of which naturally express HLA-Cw1, following metabolic labeling and two different chase periods (15 and 120 min) in the absence and presence of aspecific Cw1 ligand (11). Regardless of the cell line and length of chase, all the heavy chains, except a minor fraction of HLA-A2 components (presumably stabilized by endogenous signal peptides (37)), melted at 37 °C (Fig. 6, compare lanes 2 and 3, 5 and 6, 9 and 10, and 12 and 13), as expected. However, HLA-Cw1 (arrowheads) was specifically stabilized by its ligand (compare lanes 2 and 4, 5 and 7, 9 and 11, and 12 and 14). We conclude that although a

FIGURE 4. Molecular dynamics simulations of HLA-B15, -B46, -Cw1, and -B27 isolated heavy chains. Molecular dynamics simulations were carried out at 300 K as described under “Experimental Procedures.” The starting conformations are shown on the left as ribbon diagrams. Conformations from the final stages of the simulations were energy-minimized, and the resulting models are shown on the right. The gray regions of the ribbons correspond to amino acids 66–76, which contain, in HLA-B46 and HLA-Cw1, the KYRV motif. The potential energy variations observed during the time scale of the molecular dynamics simulations are plotted over the arrows and indicate that at 300 K, but also at 320 K (data not shown), the HLA-B46 and HLA-Cw1 models achieved a stable overall conformation at intermediate potential energy, which did not occur with the HLA-B15 or HLA-B27 model.

The KYRV 66–76 Motif in the α1-Domain α-Helix Is Involved in Strong TAP/Tapasin Association—Neisig et al. (9) have shown that HLA-C molecules accumulate, free of peptide, in association with TAP. We therefore tested whether the KYRV motif is involved in determining this feature. To this end, we used antibodies to TAP1 to compare the amounts of co-immunoprecipitated heavy chains in CHAPS extracts of 221.Cw1,
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Quantitative assessment of peptide receptivity in vivo is beyond the scope of in vitro assembly experiments, no solubility folding impairment prevents HLA-Cw1 from becoming peptide-receptive.

Discussion

Several explanations have been offered for the low surface expression of HLA-C: (a) transcript instability (10); (b) poor assembly with β2m, resulting in the accumulation of β2m-free heavy chains (8); and (c) selective peptide binding resulting in prolonged TAP association of the heavy chain, followed by endoplasmic reticulum degradation (9). Our results provide further evidence for explanation c and imply additional redundancy in the down-regulation of HLA-C expression, i.e. (d) increased dissociation of HLA-C/β2m complexes. We also show that explanation d, like explanation c, depends on an HLA-C-specific KYRV motif (5) of the α1-domain α-helix. Additionally, the KYRV motif may impair the flexibility of the class I heavy chain, as measured by molecular dynamics simulation, and confers binding not only to TAP but also to tapasin, although the acquisition of a peptide-receptive state by HLA-Cw1 requires neither TAP nor tapasin. Below, we argue that the KYRV motif introduces a single bottleneck impairing HLA-C assembly.

Impaired Assembly, Increased Disassembly, and the KYRV 66–76 Motif of HLA-C—Early studies by Neefjes and Ploegh (8) identified an abundant accumulation of free HLA-Cw2, -Cw3, and -Cw4 heavy chains. In contrast, McCutcheon et al. (10) did not find evidence for impaired assembly of HLA-Cw3, -Cw6, and -Cw7 complexes and found that assembled HLA-A2, -B27, and -Cw1 complexes are equally long-lived. Taking advantage of the distribution of the L31 epitope on a set of alleles that includes one member from each of the eight serologically defined HLA-C specificities and five HLA-B alleles, we have been able to carry out a homogeneous comparison of the accumulation of the different unfolded heavy chains and to estimate their assembly abilities. This panel is representative of the different assembly efficiencies of class I alleles because it includes HLA-B51, known as one of the poorest class I assemblers (15). At variance with McCutcheon et al., we have found (Figs. 1–3) that HLA-C heavy chains are poor assemblers and that few HLA-C heavy chain/β2m complexes (eight alleles tested), but most HLA-A and HLA-B complexes (11 alleles tested, including the HLA-A2 and HLA-B27 alleles tested by McCutcheon et al.), survive a 5-h chase. The reasons for these discrepancies are unclear. Our results are more similar to those of Neefjes and Ploegh (8) and Gillet et al. (38). The latter group observed a greater dissociation rate of HLA-B and HLA-C compared with HLA-A molecules, but because of the lack of suitable reagents such as HLA-C transfectants and antibodies, could not detect differences in dissociation rates between HLA-C and HLA-B.

HLA-B*4601 results from a gene conversion event that replaced the α1-domain α-helix of HLA-B*1501 with that of HLA-Cw*0102 (this includes both an HLA-C-specific motif and the L31 epitope). Analysis of the published (13) high pressure liquid chromatograms of eluted peptides reveals that HLA-B*4601 lost the “humped” profile of the natural HLA-B*1501 peptide ligands and acquired a “flat” profile dominated by a limited number of distinct prominent peptide peaks more similar to HLA-Cw*0102. These results are consistent with selective peptide binding by HLA-C (9) being due, at least in part, to HLA-C-specific sequences.

In this study, using the same 221.B*4601 transfectants, we have shown that the presence of an HLA-C-specific sequence confers to the interlocus recombinant several characteristics typical of HLA-C heavy chains, including local α1-domain unfolding, impaired assembly, strong binding to TAP and tapasin, and post-assembly instability. Thus, the HLA-C-specific KYRV motif (5) of the α1-domain α-helix is involved in determining a single locus-specific biosynthetic bottleneck responsible for several biochemical features of HLA-C. In contrast, the poor assembly of HLA-B51 has been mapped to the α2-domain, to residues for the most part outside the binding groove (15). Substitutions at residues 74 and 116 and at or around resi-
dues 122, 134, and 136 in the α1- and α2-domains are known to affect the association of heavy chains with members of the peptide-loading complex and transport to the cell surface (39–44). In the context of the available information, our results indicate that TAP and tapasin (presumably when associated within the peptide-loading complex) either bind an extended region encompassing the top of the α1- and α2-domain α-helices or, irrespective of their precise docking sites, sense global changes in the conformation of the entire binding groove.

Selective Peptide Loading of HLA-C—At least three groups have proposed that HLA-C selectively binds peptides (5, 9, 45). An indirect argument supporting this view is based on the observation that peptides containing certain HLA-C motifs are poorly transported by TAP, presumably resulting in the availability of a restricted pool of HLA-C ligands in the endoplasmic reticulum (discussed and referenced in Ref. 9). An alternative, although nonexclusive, mechanism for peptide selectivity was suggested by in vitro assembly experiments in which HLA-C, compared with HLA-A and HLA-B, required 10-fold greater concentrations of an equimolar mixture of completely degenerated random nonamer peptides to become peptide-bound and to be released from TAP (9). This latter experiment implies that intrinsic features of the HLA-C heavy chains restrict its ability to bind peptides, although the exact mechanism constraining ligand/acceptor combinations remains unclear.

Molecular dynamics simulation experiments (in the absence of peptide and β2m) suggest that KYRV renders the binding groove more rigid. This is in agreement with the entire set of experimental data shown here. The persistence of free heavy chains bearing linear epitopes in the α1-domain α-helix and in the α3-domain is consistent with unfolding proximal and distal to the KYRV motif (Figs. 1A and 5). Thus, local folding coupled to binding, as is believed to occur in HLA class I molecules (46), may be more demanding in KYRV-containing alleles. On this basis, the simplest interpretation of selective peptide binding is that the KYRV motif, by limiting the plasticity of the HLA-C heavy chain, restricts the range of acceptable ligands and causes the retention by TAP and tapasin of peptide-receptive heavy chains awaiting the “right” peptides. This single biosynthetic bottleneck would then cause a backward jam of irreversibly (11) unfolded heavy chain intermediates.

It should be noted that this bottleneck does not involve an absolute impairment in the ability of HLA-C to acquire a generic fold compatible with peptide binding. On the contrary, in vitro assembly experiments show that this property is conserved even in the absence of TAP or tapasin in all class I heavy chains (16, 29–35), including HLA-C (Fig. 6). Thus, our results favor a stringent constraint/selection imposed by the KYRV motif on peptide selection.

It may be of interest to determine whether or not the locus-specific biosynthetic bottleneck, described here, favors the loading of a dedicated set of peptides in the trophoblast, where HLA-C is selectively expressed in the absence of HLA-A and HLA-B, activates decidual NK cells, and favors implantation through blood vessel remodeling (47).

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