Low HLA-C Expression at Cell Surfaces Correlates with Increased Turnover of Heavy Chain mRNA

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Summary

In comparison with HLA-A and -B, the protein products of the HLA-C locus are poorly characterized, in part because of their low level of expression at the cell surface. Here, we examine how protein–protein interactions during assembly and regulation of the mRNA level affect cell surface expression of HLA-C. We find that intrinsic properties of the HLA-C heavy chain proteins do not correlate with low cell surface expression: HLA-C heavy chains associate and dissociate with β2-microglobulin (β2m) at rates comparable to those found for HLA-A and -B, and increased competition for β2m does not alter the surface expression of HLA-C. From studies of chimeric genes spliced from the HLA-B7 and -Cw3 genes, we find that chimeric proteins containing the B7 peptide-binding groove can have low cell surface expression, suggesting that inefficiency in binding peptides is not the cause of low cell surface expression for HLA-C. The surface levels of HLA-A, -B, or -C in cells transfected with cDNA can be similar, implicating noncoding regions of HLA-C heavy chain genes in the regulation of surface expression. We find that HLA-C mRNA is expressed at lower levels than HLA-B mRNA and that this difference results from faster degradation of the HLA-C message. Experiments examining chimeric B7/Cw3 and B7/Cw6 genes suggest that a region determining low expression of HLA-C is to be found between the 3′ end of exon 3 and a site in the 3′ untranslated region, ~600 bases downstream of the translation stop codon.

H LA class I molecules can be found on the surface of most types of human nucleated cells; they function in the presentation of endogenously processed peptide antigens to CTLs (reviewed in reference 1) and the inhibition of NK cells (reviewed in reference 2). The products of the HLA-A and -B loci have been well characterized in terms of their serology, structure, and function (3–5). In contrast, the serology of HLA-C molecules remains crude, which in turn has impeded analysis of the structure and function of HLA-C (6). Despite these limitations, HLA-C molecules have been shown to present peptides to CTLs (7) and to inhibit NK cells (8).

In most human populations, the products of 10–50% of the HLA-C alleles are not recognized by the human alloantisera used in HLA typing and are therefore defined as serologically “blank” alleles (9). However, biochemical analysis (10) and DNA cloning (11, 12) demonstrate that the blank alleles are associated with functional genes and that the failure of human alloantisera to recognize HLA-C blanks is not caused by the absence of a functional HLA-C molecule at the cell surface.

Snary et al. (13) reported in 1977 that HLA-C molecules are expressed at the cell surface at levels much lower than either HLA-A or -B. Since then, other groups of investigators have suggested possible causes for the low surface expression of HLA-C molecules. One view is that intrinsic properties of HLA-C heavy chain polypeptides are responsible (6). By analogy with H-2Ld, a mouse class I molecule with relatively low expression at the cell surface, HLA-C heavy chains might associate inefficiently with β2-microglobulin (β2m) and peptide in the endoplasmic reticulum (14). Alternatively, HLA-C molecules could dissociate more rapidly. In this vein, Neefjes and Ploegh (15) have postulated a decreased affinity of HLA-C heavy chains for β2m, owing to HLA-C–specific residues of the α3 domain.

A second view holds that low HLA-C expression results from factors affecting HLA-C mRNA and not from intrinsic properties of the HLA-C heavy chain. Shimizu and DeMars (16) found that levels of HLA-C mRNA in transfected cells

1 Abbreviations used in this paper: β2m, β2-microglobulin; 1D-IEF, one-dimensional isoelectric gel focusing electrophoresis; HAT, hypoxanthine aminopterin thymidine; UTR, untranslated region.
were lower than those for HLA-A or -B. On the other hand, Tibensky et al. (12) found similar mRNA levels for HLA-B and -C in EBV-transformed B cell lines. Several different enhancer and promoter elements are present in HLA-A, -B, and -C genes, but sequence comparisons revealed no correlation between the presence of particular elements and the level of cell surface expression (17, 18). However, no direct comparison of HLA-A, -B, and -C promoters using reporter gene constructs has been performed, so the role of pretranslational regulation remains unclear.

In summation, there exists a confusing and contradictory literature pertaining to the low cell surface expression of HLA-C molecules and as yet there is no consensus as to the underlying mechanism. The experiments described here were undertaken to help clarify these issues.

Materials and Methods

Nomenclature. HLA-A, -B, and -C are designated by serological assignment. Where appropriate, the official allele designations are given in parentheses (19, 20).

Cell Culture. Cell lines were cultured in RPMI 1640 (Mediatech, Washington, DC) containing 10% BSA (Hyclone, Logan, UT), as previously described (21). Jesthomi (A2, B27, Cw1), JY (A2, B7, Cw7), JAP NF (A2;26, B38;63, Cw7) (22), KT 17 (A2;11, B35;62, Cw3;4) (22), GEE018 (A2;29, B7;76, Cw6) (23), GRC187 (A28;31, B35;62, Cw3) (24), and Olga (A31, B62, Cw11) (22) are EBV-transformed B cell lines. Mutant EBV cell lines we used are 721.221, which is HLA-A, -B, and -C negative (16); 721.184, which expresses Cw4 (Cw*0401) and a small amount of B35;62, Cw3;4) (22), GEE018 (A2;29, B7;76, Cw6) (23), GRC187 (A28;31, B35;62, Cw3) (24), and Olga (A31, B62, Cw11) (22) are EBV-transformed B cell lines. Mutant EBV cell lines we used are 721.221, which is HLA-A, -B, and -C negative (16); 721.184, which expresses only the HLA-Cw1 gene (16); and HmyCIR (CIR), which expresses Cw4 (Cw*0401) and a small amount of B35 (B*3501) (25).

Transfection with Genomic DNA. Genomic clones for HLA-A2 (A*0201), B7 (B*0702), B7 (B*2705), Cw3, Cw6, and Cw7 in the pHEBo shuttle vector were transfected into the 721.221 cell line (16) as previously described (21). 721.221 cells transfected with the pHEBo shuttle vector required no selection by flow cytometry, as they give a unimodal distribution when analyzed by flow cytometry after staining with a class I HLA-specific mAb. Unsorted hygromycin-resistant cells were therefore pooled and expanded. At least two separate transfections with each chimeric gene were assayed. Transfectants were maintained in medium containing 300 μg/ml hygromycin B (Boehringer Mannheim Biochemicals, Indianapolis, IN) throughout the culture period.

In experiments in which the effect of competition for βm between either HLA-C or -B and -A2 heavy chains was examined, the B7, Cw3, Cw6, or Cw7 gene was transfected into a recipient cell, which is itself a transfectant of 721.221 expressing HLA-A2 in a stable fashion. This recipient cell was made by transfection of the HLA-A2 gene (subcloned in the shuttle vector pHPT32) into 721.221 cells and selection using hypoxanthine aminopterin thymidine (HAT) medium (Sigma Chemical Co., St. Louis, MO). A pool of HAT-resistant cells were then sorted for HLA-A2 expression using a FACStar® Plus flow cytometer (Becton Dickinson, San Jose, CA), and the sorted populations were cultured further. The cell line thus obtained has maintained expression of HLA-A2 during continuous culture in medium lacking HAT for 6 mo. In making "double" transfecteds using this recipient cell line, the second class I HLA gene was introduced using the pHEBo vector and selection with hygromycin B as described in the previous paragraph.

Transfection with cDNA. HLA-A, -B, and -C cDNAs were isolated from EBV-transformed cell lines and characterized as described by Domena et al. (26). Clones isolated by these methods contain only a portion of the 3' untranslated region (UTR) because the 3' primer used for PCR amplification of the cDNA binds to a sequence within this region (Fig. 1, insert 10). Sequenced cDNA clones were subcloned into the integrating expression vector pB1 neo(27) (provided by M. Davis, Stanford University, Stanford, CA) using Sal and HindIII sites within the cDNA and HindIII and XhoI sites within the vector. cDNA constructs were transfected using a gene pulser (Bio Rad Laboratories, Richmond, CA) set at a field strength of 625 V/cm at 960 μF. After transfection, the cDNAs were cultured under G418 selection (1 mg/ml; Gibco BRL, Gaithersburg, MD), which led to discrete populations of class I-positive and class I-negative G418-resistant cells. The G418-resistant cells were sorted using a FACStar® Plus flow cytometer to isolate the population of HLA class I-positive cells. Sorting the cDNA transfectants was found not to increase the class I expression level of the class I-positive population, but merely to eliminate the population of class I-negative cells.

Construction of Cw3/B7 and B7/Cw6 Chimeric Genes. The p158 plasmid containing the B7 genomic clone was obtained from J. Barbosa (Harvard University, Boston, MA), and the pCw3 plasmid containing the Cw3 genomic clone (28) was from H. Ploegh (Massachusetts Institute of Technology, Cambridge, MA). B7pHEBo was generated as described (21). Cw6pHEBo and Cw7pHEBo were provided by D. Schendel (Institute for Immunology, Munich, Germany). DNA containing the EcoRI-XbaI 4.6-kg fragment of Cw3 was cloned into the pHEBo plasmid to generate Cw3pHEBo. To confirm that the gene downstream of the polyadenylation signal of B7 did not contain a control element, we made a construct, B7A, in which the region 3' to the BstII site was deleted in the B7 gene (Fig. 1, insert 2). The chimeric genes included Cw3E/B7, the Cw3 enhancer fused to the remainder of the B7 gene using EcoRI and XbaI (Fig. 1, insert 5); Cw3pL/B7, the B7 enhancer fused to the Cw3 promoter through exon 1, fused to the remainder of the B7 gene using XbaI and a PCR primer 5'-GGCGGGGTCTCAGCCCCC; B7/Cw3, the B7 enhancer element fused to the Cw3 gene using EcoRI and XbaI (Fig. 1, insert 7); B7Ex3/Cw3, the B7 enhancer through exon 3 fused to the remainder of the Cw3 gene using EcoRI and KpnI (Fig. 1, insert 8); and B7Ex3/Cw6, the B7 enhancer through exon 3 fused to the remainder of the Cw6 gene using EcoRI and KpnI (Fig. 1, insert 9).

All but one of the chimeric genes were generated by ligating restriction endonuclease fragments according to standard procedures. The exception, Cw3pL/B7 (Fig. 1, insert 6), was made using a two-step PCR protocol (29, 30) and contains the Cw3 sequence from the XbaI site in the promoter through most of intron 1. All PCR-amplified and cloned DNAs were sequenced to confirm the recombinations and identify artifactual mutations. In doing this, we discovered differences from the sequence reported by Sodoyer et al. (28) for this very same genomic clone. In the promoter, we found the CCAAT box (at 73–77 nucleotides upstream of the initiation methionine codon) to have the canonical CCAAT sequence, not CCGGT as described previously. Furthermore, the coding sequence matched Cw*0302 and not the Cw*0301 sequence determined by Sodoyer et al. (28). Thus the nucleotide substitutions that distinguish C*0301 and C*0302 may all be the result of errors in the C*0301 sequence.

Flow Cytometry. Surface expression of class I HLA molecules was measured by flow cytometry using a FACScan® flow cytometer (Becton Dickinson); data analysis was performed using a VAX computer and the Electric Desk program (Herzenberg Laboratory, Stanford University). Approximately 5 x 10^6 cells were incubated...
with antibodies as described (21), except that 2 μg of purified antibody was used in the primary incubation. For analysis of the cDNA transfectants, 2 μg of purified W6/32 conjugated to fluorescein was used. In each experiment, mAb binding to untransfected 721.221 cells (background binding) was subtracted from mAb binding to the transfectant. Cells transfected with chimeric genes were assayed in at least two separate experiments, using two or more broadly reactive mAbs.

Isoelectric Focusing. Cells were metabolically labeled with [35S]Met/Cys (Amersham Corp., Arlington Heights, IL), immunoprecipitated, and treated with neuraminidase as described (31). One-dimensional isoelectric focusing gel electrophoresis (1D-IEF) was performed as described (31). The gels were exposed to film or to a Phosphorscreen (Molecular Dynamics, Inc., Sunnyvale, CA). Class I heavy chain isoforms were quantitated using ImageQuant (Molecular Dynamics, Inc.). The three isoforms of Cw1 were considered together.

Half-Life Determination. The half-lives of HLA-A, -B, and -C genes were determined using the formula \( k = \frac{T_1}{\log(V_2) - \log(V_1)} \), where \( k \) is the half-life, \( T \) is the time, and \( V \) is the density of the isoform. The linear part of the curve (4–40 h) was used to generate the \( r^2 \) value for each data set by linear regression: \( r^2 \) values were 0.932, 0.991, and 0.776 for HLA-A, -B, and -Cw1, respectively.

RNA Preparation. Total cellular RNA was prepared from 2 × 10⁷ cells using RNAzol (32) according to the manufacturer’s instructions (Tel-Test Inc., Friendswood, TX).

DNA Labeling and Oligonucleotide Specificity. To obtain probes for Northern blot analysis, oligonucleotides that bound to the homologous sites in the transmembrane domain of either the HLA-B or -C sequence and have similar G/C content were designed. 100 pmol of either the “HLA-B-specific” oligonucleotide (5'-AGC-TCCGATGACCACAACATC) or the “HLA-C-specific” oligonucleotide (5'-AGCTTCTAGGACGCTCAGAC), where boldface letters indicate the nucleotide differences, was end labeled with [γ-32P]ATP (Amersham Corp.) using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals). Labeled oligonucleotides were separated on a Sephadex G50 column (Pharmacia LKB Biotechnology Inc., Alameda, CA). 1 ml of each sample was assayed using a scintillation counter (LS5000CE; Beckman Instruments, Fullerton, CA), and an equivalent amount of radioactivity for either the HLA-B-specific or -C-specific oligonucleotide was added to each hybridization mixture.

To demonstrate the specificity of the oligonucleotides, samples of total cellular RNA isolated from 721.221 transfectants expressing the A2, B7, B27, Cw3, Cw6, or Cw7 gene and from the mutant cell line 721.184, which expresses a single copy of the Cw1 gene, were analyzed on a Northern blot. The oligonucleotide designed to be HLA-B-specific bound RNA from the B7 and B27 transfectants, but not RNA from any other transfectant or mutant cell lines. The oligonucleotide designed to be HLA-C-specific bound RNA from the Cw3, Cw6, and Cw7 transfectants and 721.184, but not from the other cell lines. This assay demonstrated that the two oligonucleotides distinguish HLA-B and -C mRNA.

To demonstrate that the radiolabeled oligonucleotides had equivalent specific activity, we analyzed the binding of the HLA-B- and -C-specific oligonucleotides to 150–300 fmol of B7 and Cw3 plasmid DNA and quantitated the binding using a PhosphorImager (Molecular Dynamics, Inc.). In three separate experiments, the amount of HLA-B-specific oligonucleotide bound to B7 DNA was...
HLA-A or -B, or -C, and expresses single epitopes of cell line 721.221, which is HLA-A, -B, and -C negative. We reinvestigated the question of HLA-C expression, and eliminated this variable by using mAbs that react with all HLA-A, -B, and -C products and cell lines expressing single HLA-A, -B, or -C alleles. Among the cells examined were transfec-
tants expressing single HLA-A, -B, or -C alleles. C1K, a mutant cell line that expresses Cwl*0401 and a small amount of Cw3 DNA.

Clone HHCSA65 (American Type Culture Collection, Rockville, MD), an 18S ribosomal cDNA, was used as a positive control on Northern blots (33). This probe was labeled with [32P]CTP using random oligonucleotide primers (Boehringer Mannheim Biochemicals). Labeled cDNA was separated from other reaction products on a Sephadex G50 column.

**Northern Blot Analysis.** 20 µg of total RNA was loaded onto each lane of a formaldehyde gel (34). Each assay was performed in duplicate. After electrophoresis, the RNA was transferred onto a Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) using 10× SSC downward transfer (35). After wetting and prehybridization, 2–4 × 10⁶ cpm of labeled HLA-B or -C-specific oligonucleotide was added to the hybridization mixture and incubated overnight at 55°C. After hybridization, the membranes were washed twice at room temperature in 1× SSC, 0.1% SDS, followed by a 1-h wash at 55°C in 1× SSC, 0.1% SDS. The binding of each oligonucleotide was analyzed on a PhosphorImager. After quantification of mRNA, the membranes were stripped by washing them at 90°C in 0.1% SDS for 16 h and then reanalyzed using 1–4 × 10⁶ cpm of the 18S ribosomal probe and the same conditions for hybridization and washing.

**Quantitation of mRNA Levels.** After incubation on a Phosphor-screen, the amount of RNA in each lane was calculated, and the background radioactivity was subtracted. The amount of mRNA in each lane was normalized to the amount of 18S ribosomal RNA in the same lane. Data are presented as a percentage of ribosomal RNA.

**Actinomycin D Treatment and Half-Life Determination.** Actinomycin D was added at 4–6 mg/5–9 × 10⁶ cells/µl (36). Aliquots of 10⁶ cells were collected from actinomycin D–treated and untreated control samples at times from 0 to 8 h after initiation of treatment. Generally, the levels of RNA became undetectable after 3–5 h of treatment, depending upon the experiment. 10–20 µg of RNA was loaded on each lane, and each sample was assayed in duplicate. After quantification using the HLA-B or -C-specific oligonucleotide, the membranes were stripped and reprobed with the 18S ribosome probe. 18S ribosomal RNA was selected as the control because it is unaffected by actinomycin D treatment (37). After normalizing the amount of HLA-B or -C message to the amount of 18S ribosomal RNA in each lane, the half-life of each message was calculated according to the formula k = T1/2(log2(Vf)) – log2(Vi), where k is the half-life, T is the time, and Vi is the normalized value of the message. r² values were calculated to be 0.999 for the HLA-B message and 0.976 for the HLA-C message.

**Results**

**HLA-C Molecules Have Lower Cell Surface Expression than HLA-A or -B.** Using quantitative serology, Snary et al. (13) found the amount of HLA-C protein expressed by lymphocytes to be ~10% that of either HLA-A or -B. The validity of this comparison has been uncertain because different alloantisera were used to quantitate HLA-A, -B, and -C. In investigating the question of HLA-C expression, we eliminated this variable by using mAbs that react with all HLA-A, -B, and -C products and cell lines expressing single HLA-A, -B, or -C alleles. Among the cells examined were transfec-
tants of cell line 721.221, which is HLA-A, -B, and -C negative and expresses single HLA-A, -B, or -C alleles; CIIR, a mutant cell line that expresses Cw*0401 and a small amount of B*3503; and 721.184, which expresses Cw1 as the only HLA-A, -B, and -C product.

Cell surface expression was compared by an indirect binding assay using flow cytometry. Results obtained with the monomorphic W6/32 antibody are shown in Table 1. Comparable levels of binding were seen for 721.184, CIIR, and 721.221 cells transfected with Cw3, Cw6, or Cw7 genomic clones in the pHEBo expression vector. The levels of HLA-C in these cells were 15–35% of those seen for genomic pHEBo transfectants expressing single HLA-A or -B alleles. In these experiments, transfected cells were selected for resistance to hygromycin B and not for levels of class I HLA expression. Thus the relative levels of HLA-A, -B, or -C expression were not biased selectively by the transfection procedure. The levels of class I expression observed for the genomic HLA-A and -B transfectants of 721.221 were comparable to those found for "normal" EBV-transformed B cell lines, consistent with the results of Sugden et al. (38) and indicating that expression of HLA-A, -B, and -C from genomic DNA constructs in the transfected mutant cells approximates that of EBV-transformed B lymphocytes. (Similar results were obtained from experiments using five mAbs against monomorphic epitopes different from those recognized by W6/32.) Our results, consistent with those of Snary et al. (13), show that cell surface expression of HLA-C is considerably lower than either HLA-A or -B expression.

**HLA-A, -B, and -C Molecules Have Similar Kinetics of Association and Dissociation with β2m.** A possible cause of low surface expression is a slow rate of association of HLA-C heavy chains with β2m. We examined the rate of association of HLA-A, -B, and -C heavy chains with β2m in pulse-chase experiments. B cell lines were radiolabeled metabolically for 30 or 60 s, followed by addition of excess nonradioactive amino acids. HLA-A, -B, and -C heavy chains associating with β2m were then isolated using mAb W6/32, which is specific for the complex, whereas free HLA-B and -C heavy chains were isolated using HC10, an mAb specific for free heavy chains. The immunoprecipitates were analyzed by 1D-IEF, which permits direct comparison of the heavy chain allootypes (Fig. 2). B7, B27, and Cw7 associated with β2m during the 30-s labeling, showing that at least one HLA-C molecule can associate rapidly with β2m.

During the 30-s period of labeling, β2m associated with the A2 heavy chains in JY cells, but not those in Jesthom cells. Even after a 60-s labeling period, neither the A2 nor the Cw1 heavy chains in Jesthom cells associated with β2m. In contrast, the B27 heavy chain in Jesthom cells associated with β2m within 30 s. These data show how the rate of association for the same class I heavy chain can vary among B cell lines.

Experiments in which 5-min labelings were followed by chase periods of various length demonstrated association of A2, A28, B53, Cw4, and Cw6 heavy chains with β2m within 5 min, whereas Cw1 required 15 min (data not shown). Thus, we find that HLA-C heavy chains associate with β2m in a time frame similar to that observed for HLA-A and -B heavy chains. From these experiments, we also determined that a cohort of Cw4, Cw6, and Cw7 heavy chains com-
Table 1. Cell Surface Expression of Class I HLA Molecules

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>C1R*</td>
<td>18(24)</td>
</tr>
<tr>
<td>721.184*</td>
<td>24(32)</td>
</tr>
<tr>
<td>Cw3^1</td>
<td>22(30)</td>
</tr>
<tr>
<td>Cw6^1</td>
<td>25(34)</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>B27^1</td>
<td>69(93)</td>
</tr>
<tr>
<td>B7A^1</td>
<td>ND</td>
</tr>
<tr>
<td>Cw3E/B7^1</td>
<td>70(95)</td>
</tr>
<tr>
<td>Cw3PL/B7^1</td>
<td>68(92)</td>
</tr>
<tr>
<td>B7E/Cw3^2</td>
<td>19(26)</td>
</tr>
<tr>
<td>B7E-ex3/Cw3^1</td>
<td>21(28)</td>
</tr>
<tr>
<td>B7E-ex3/Cw6^1</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mutant EBV-transformed B lymphocyte cell lines. C1R expresses Cw*0401 and a very low level of B*0303; 721.184 expresses Cw1 as the only class I molecule.

1 Transfectants of the cell line 721.221, which is negative for HLA-A, -B, and -C. Each cell line is named for the transfected genomic DNA. B7D is the truncated B7 molecule shown in Fig. 1, line 2, Cw3E/B7 is the chimera shown in line 5, Cw3PL/B7 is the chimera shown in line 6, B7E/Cw3 is the chimera shown in line 7, B7E-ex3/Cw3 is the chimera shown in line 8, and B7E-ex3/Cw6 is the chimera shown in line 9.

2 In each experiment, binding of mAb W6/32 to untransfected 721.221 cells was subtracted from W6/32 binding to the tested cell. The values of W6/32 binding to untransfected 721.221 cells were 5, 7, 7, 6, and 6 fluorescence units in experiments 1, 2, 3, 4, and 5, respectively. Data were collected on a four-decalogue scale.

3 In parentheses, the W6/32 binding is shown as a percentage of the binding obtained with the HLA-B7 transfectant in the same experiment.

ND, not done.

In conclusion, this analysis indicates that slow or incomplete association with β2m within 1 h (data not shown). In another potential cause of low expression is that HLA-C molecules have a shorter half-life than HLA-A and -B, a mechanism that accounts for the reduced expression of H-2D^d relative to other mouse class I molecules (39). To compare the dissociation kinetics, we performed additional pulse-chase experiments. Jythom cells were labeled for 15 min and then chased with nonradioactive amino acids over a 5-d period. HLA-A, -B, and -C heavy chains were immunoprecipitated with W6/32 and analyzed on a 1D-IEF gel. By quantitating the amount of heavy chains remaining in the W6/32-recognizable form during the chase periods, we estimated the half-life for each allotype. The half-lives for A2, B27, and Cw1 were 18, 30, and 15 h, respectively (Fig. 3); these results show that Cw1 does not dissociate significantly faster than A2. Thus, a short half-life is probably not the cause of the low surface expression for HLA-C molecules. Overall, these data do not support the view that HLA-C heavy chains have, as a group, slow association and/or rapid dissociation kinetics compared with their HLA-A and -B counterparts.

Competition for β2m Does Not Alter the Surface Expression of HLA-C Molecules. Neefjes and Ploegh (15) have suggested that low surface expression of HLA-C molecules derives from poor affinity of the HLA-C heavy chains for β2m. If HLA-C heavy chains bind poorly to β2m, then transfectants containing a single HLA-C gene should have higher surface expression than transfectants containing both an HLA-A and
Figure 3. A log–log plot showing the decay of A2, B27, and Cw1 molecules. Jethom cells were pulse labeled with [35S]methionine and then chased with nonradioactive methionine for 1–5 d. At each time point, aliquots of cells were lysed, and the class I HLA molecules were immunoprecipitated with mAb W6/32. The immunoprecipitates were treated with neuraminidase to remove sialic acid residues and were analyzed by 1D-IEF. After exposure to a Phosphorscreen, the samples were analyzed and quantitated on a PhosphorImager.

HLA-C gene. We performed experiments to examine the capacity of HLA-C heavy chains to bind β2m in the presence and absence of competing HLA-A2 heavy chains. Cell surface expression of the HLA-C and -A2 molecules was measured using indirect antibody binding and flow cytometry. The surface expression for HLA-C molecules was detected using mAb 4E, which recognizes HLA-B and -C molecules but not HLA-A2 molecules (40); the surface expression of

Figure 4. Comparison of the surface expression of A2, B7, Cw3, Cw6, and Cw7 molecules on singly and doubly transfected cells as assayed by flow cytometry. The expressed class I molecules are as indicated under each cell line. The surface expression of class I molecules was assessed using a four deca-logue scale. The y axis shows the change in antibody binding. The change in fluorescence was calculated by subtracting the fluorescence obtained by staining the untransfected 721.221 cell line with the MA2.1 or the 4E antibody from the fluorescence obtained by staining transfectants with the same antibody. Unshaded bars show MA2.1 binding; black bars show 4E binding. Values of MA2.1 binding to the B7, Cw3, Cw6, and Cw7 transfectants and of 4E binding to the A2 transfectant were the same as binding to 721.221 cells.
A2 was detected using mAb MA2.1, which is specific for A2 and B17 (41). We found that transfected cells expressing a single HLA-C gene have the same level of surface expression as those expressing A2 in addition to the HLA-C gene (Fig. 4). Thus, competition for β2m by A2 has no observable effect on the cell surface expression of HLA-C. Transfectants expressing A2 alone or A2 in conjunction with B7, Cw3, Cw6, or Cw7 have similar expression levels, showing that competition for β2m by these other heavy chains does not affect the surface expression of A2 (Fig. 4). This is true for both the mean fluorescence values (Fig. 4) and the shape of the histogram (data not shown). This analysis shows that competition for β2m does not limit surface expression of HLA-C molecules, and low surface expression is therefore unlikely to stem from low affinity for β2m.

HLA-A, -B, and -C cDNAs Can Direct Comparable Expression Levels in Transfected Cells. Additional evidence that the structure of HLA-C heavy chains does not cause low cell surface expression was obtained by analysis of cDNA transfectants (Fig. 5). cDNAs for various HLA-A, -B, and -C alleles were subcloned into pBJ1-neo, an integrating expression vector, and transfected into the HLA class I-negative 721.221 cell line. The pBJ1-neo vector has a hybrid viral promoter that drives expression of the inserted cDNA (42). Additionally, the HLA class I cDNAs used in these constructs do not contain the entire 3' UTR. Thus, unlike transfection of genomic constructs, this system is not subject to locus-specific promoter or enhancer effects on gene expression.

Constructs encoding HLA-C molecules were transfected multiple times to generate independent transfected cell lines expressing the same allele. Class I expression levels of these transfectants varied (Fig. 5). Expression level differences were not correlated with particular alleles, suggesting they were more likely caused by differences in the site of integration or copy number of the transfected genes than to properties of the expressed protein product. Significantly, in this system many of the HLA-C transfectants showed expression levels equivalent to or exceeding those of HLA-A and -B transfectants. This shows that HLA-A, -B, and -C molecules can be expressed at similar levels. Furthermore, some of the HLA-C transfectants had high class I expression comparable to the total class I expression of an EBV-transformed B cell line, indicating that HLA-C proteins have the capacity to be expressed at high levels.

Low Cell Surface Expression Is Determined by a Region of the HLA-C Gene 3' to Exon 3. To identify regions of the HLA-C gene responsible for low surface expression, we made a series of chimeras between B7 and Cw3 genomic DNA clones (Fig. 1). These chimeras were subcloned into the shuttle vector pHEBo and transfected into the 721.221 cell line. Cell surface expression of the products of the transfected genes was assessed by indirect antibody binding using flow cytometry.

From analysis of the five chimeric genes, we obtained evidence suggesting that surface expression may be controlled by a region beginning at the 3' end of exon 3 and ending in the 3' UTR ~600 bases downstream from the translation stop site. The most informative comparisons are the transfectants containing the B7 enhancer fused to the remainder of the Cw3 gene and the enhancer through to exon 3 of B7 fused to the 3' part of the Cw3 gene (Table 1). Transfectants containing either of these chimeras had surface expression levels similar to those of the transfectant containing the parental Cw3 gene.

To investigate the role of a region downstream from exon 3, we generated an additional chimeric gene by fusing the enhancer through exon 3 of B7 to exons 4 through the 3' UTR of Cw6. The transfectant expressing this chimera (B7ex3/Cw6) has cell surface levels of class I molecules similar to the transfectant expressing the parental Cw6 gene, further indicating that the region controlling low surface expression is downstream from exon 3.

Exchanging the enhancer and promoter of the B7 and Cw3 genes has little effect on cell surface expression, showing that regulatory sequences in the 5' region, including the promoter of HLA-C, are not responsible for the low expression levels. These results concur with the conclusions of Tibensky et al. (18) and Steinle et al. (17). Transfectants expressing the "wild-type" B7 gene and a B7 construct truncated in the 3' UTR, 360 bases after the translation stop site, have similar expres-
sion levels, showing that the sequence of the 3' UTR downstream from the truncation in B7 has no effect on cell surface expression. In aggregate, these experiments show the low surface expression of HLA-C proteins to be determined by a region delimited by the 3' end of exon 3 to the 3' UTR, ~600 bases after the translation stop codon.

HLA-C Message Levels Are Lower than HLA-B Message Levels. Shimizu and DeMars (16) noted that the mRNA levels for transfected HLA-C genes were lower than those for either the HLA-A or -B gene. Reduction in mRNA might also explain why "normal" B cell lines express less HLA-C than either -A or -B. To address this question, we compared the amount of HLA-B and -C message by Northern blotting using radiolabeled HLA-B- or -C-specific oligonucleotides. Analysis of independent preparations of total cellular RNA from the Jesthom and JY cell lines showed the amount of HLA-B message to be between 2 and >10 times greater than the amount of HLA-C (Fig. 6a). Testing additional cell lines gave similar results (Fig. 6b). Thus, low surface expression of HLA-C is correlated with low mRNA levels.

HLA-C Message Has a Shorter Half-Life than HLA-B Message. Low surface expression of HLA-C molecules corresponds to the presence of HLA-C sequences downstream from exon 3. Assuming the low surface expression results from reduced mRNA levels, a sequence downstream from exon 3 is its most likely cause. Regulation of expression by the 3' part of a gene is usually caused by an element that destabilizes the message (37). To compare the stability of HLA-B and -C messages, we treated the cell line Jesthom with actinomycin D, an inhibitor of transcription initiation, and quantitated HLA-B and -C mRNA after different time periods of treatment by Northern blotting using locus-specific oli-
gonucleotides. The half-life for B27 mRNA was found to be ~3 h, whereas that for Cw1 was ~0.75 h (Fig. 7). Thus, HLA-C message is found to turn over more quickly than HLA-B message, resulting in a lower steady-state level of HLA-C mRNA. In turn, this is expected to reduce the rates of translation, synthesis of HLA-C heavy chains, assembly of HLA-C molecules, and ultimately their steady-state levels at the cell surface.

Discussion

We find levels of HLA-C molecules at the cell surface to be 15–35% those of HLA-A and -B, similar to the differences reported by Snary et al. (13). In B cell lines, the reduced expression of HLA-C protein correlates with a lower level of HLA mRNA. Our results are consistent with previous comparisons of HLA-A, -B, and -C mRNA levels in a variety of cell types (16, 43–46), but they contrast with those of Tibensky et al. (12), who reported similar levels in EBV-transformed B cell lines. A possible explanation for the difference is that the oligonucleotide probe used by Tibensky et al. to detect HLA-C cross-hybridized with HLA-B. The specificities of our antisense oligonucleotide probes were determined using RNA, whereas Tibensky et al. used plasmid DNA. Oligonucleotides bind more tightly to RNA than to DNA, so that the conditions established by Tibensky et al. to be specific for DNA may not have been as specific for RNA. In preliminary assessment of oligonucleotides designed to be specific for HLA-B or -C, we found several putative HLA-C-specific oligonucleotides that bound nonspecifically to RNA prepared from HLA-B transfectants. Indeed, the oligonucleotides we eventually used were the fourth pair assessed for appropriate specificity.

In contrast to our findings, Neefjes and Ploegh (15) have suggested that HLA-C heavy chains associate slowly and inefficiently with β2m. In reaching their conclusions, Neefjes and Ploegh (15) assumed that a polyclonal rabbit antisera had a similar affinity for free HLA-A, -B, and -C heavy chains as mAb W6/32 has for β2m-associated heavy chains, which may not have been the case. We find that anti-heavy chain antisera and mAb vary in their abilities to recognize heavy chain allotypes. Evidence for this phenomenon can also be seen in the 1D-IEF gels presented by Neefjes and Ploegh (15), in which more HLA-B27 heavy chain is immunoprecipitated by the anti-heavy chain antisera than by W6/32, whereas the reverse is true for HLA-A11 heavy chains. Thus, the larger amount of HLA-C free heavy chain detected by their rabbit antisera may have been a consequence more of its higher affinity for HLA-C than of the level of HLA-C translation.

Our study points to low levels of mRNA being the cause of low HLA-C expression at the cell surface. In our current working hypothesis, it is the rate of HLA-C heavy chain biosynthesis that limits cell surface expression and not intrinsic deficiencies in either the HLA-C heavy chain or peptide supply, as suggested previously (6, 15). Furthermore, the lower levels of HLA-C mRNA are due to a higher rate of degradation caused by sequences within a region in the 3' part of the HLA-C gene that begins 3' to the end of exon 3 and ends in the 3' UTR, ~600 bases downstream of the translation stop site. The fact that cells transfected with cDNA can express HLA-C at high levels indicates that the elements controlling HLA-C expression levels are not a function of the exons and is consistent with their location in introns or the 3' UTR. Given that turnover of the mature spliced mRNA is affected, the most likely location for the regulatory element is in the 3' UTR, downstream of the binding site for the oligonucleotide primers used to clone the cDNA (Fig. 1, insert 10). In HLA-B and -C sequences, the first polyadenylation signal occurs 90 or 170 bases, respectively, downstream from the end of the cDNA transcript. Thus, the HLA-C message rearranged from genomic transcripts could be 80 bases longer than the HLA-B message and could, in that additional segment, contain distinctive regulatory elements.
Little is known about the mechanisms of mRNA degradation. Caput et al. (47) have shown that a repeating octamer, UUAUUAUAA, in the 3′ UTR destabilizes cytokine mRNA. The presence of A/U-rich regions destabilizes the binding of the poly(A)-binding protein (48). Moreover, c-myc and c-fos share an A/U-rich region with cytokine genes, although both protooncogenes have additional destabilizing elements within the translated protein (49, 50). HLA-C genes do not have A/U-rich sequences in their 3′ UTRs and may not be regulated by mechanisms similar to those found for cytokines and protooncogenes. Clearly, future experiments should be directed at a more precise definition of the regulatory elements destabilizing HLA-C mRNA.

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