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Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid

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Biosynthesis of HLA class I antigens has been studied in a variant B-LCLxT-LCL hybrid, 174XCEM.T2. This cell line encodes HLA-A2 and -B5, but expresses only small amounts of A2 antigen and undetectable B5 antigen at the cell surface due to a mutation inactivating a *trans*-acting regulatory gene encoded within the class II region of the human major histocompatibility complex. Northern blot analysis with HLA-A- and HLA-B-specific probes shows that 174XCEM.T2 synthesizes quantities of A and B locus mRNA comparable with its class I antigen-positive parent cell line. Immune precipitation studies indicate that 174XCEM.T2 synthesizes normal HLA heavy chains and β_2-microglobulin which fail to form dimers. The heavy chains are N-glycosylated normally, but processing of the glycan to the complex form does not occur. In addition, free heavy chains in this cell line are not phosphorylated. Thus, the majority of class I heavy chains in 174XCEM.T2 do not combine with β_2-microglobulin, and are not processed or transported to the cell surface. As both subunits are synthesized in normal amounts, we propose that an additional molecule absent from 174XCEM.T2 and encoded by an HLA-linked gene is necessary for efficient assembly of class I antigen subunits.

Key words: HLA antigens/MHC/membrane glycoproteins/protein transport

Introduction

The major histocompatibility complex (MHC) encodes a series of highly polymorphic membrane-bound glycoproteins of central importance to the immune system. The human class I antigens, consisting of HLA-A, -B or -C heavy chains non-covalently associated with β_2-microglobulin (β_2m), must be expressed at the surface of allogeneic or virus-infected cells for their recognition and lysis by cytotoxic T lymphocytes (McMichael *et al.*, 1977). Structurally, the heavy chain is comprised of three globular domains, each of ~90 amino acids, on the external side of the plasma membrane, followed by a stretch of hydrophobic amino acids spanning the lipid bilayer and a short cytoplasmic tail at the C terminus (reviewed in Ploegh *et al.*, 1981). The N-terminal α1 domain is highly variable in amino acid sequence and contains most of the alloantigenic determinants. A single N-linked complex carbohydrate side chain is attached at position 86 in the α1 domain. The α2 and α3 domains contain disulfide loops of ~60 amino acids, and the α3 domain bears structural homology to immunoglobulin constant region domains. Experiments in the mouse have shown that β_2m associates with the heavy chain of the homologous H-2 glycoprotein via the α3 domain (Yokoyama and Nathenson, 1983).

Although class I molecules are expressed on most nucleated cells of the body, regulation of expression of these antigens has been uncovered in a number of cell types. Cell lineages which are deficient in surface expression of these antigens include neurons (Lampson *et al.*, 1983), mature trophoblasts (Faulk *et al.*, 1977), and malignancies such as embryonal carcinomas (Trowsdale *et al.*, 1980), neuroblastomas (Lampson *et al.*, 1983), small cell lung cancers (Doyle *et al.*, 1985) and some T lymphoblastoid cell lines (T-LCL) (Billings and Lucero, 1982; Howell *et al.*, 1984). Northern blot analysis of these cells has shown that they express low levels of class I mRNA. Other cells which express low levels of class I antigens can be induced to increase expression by treatment with interferons (reviewed in Rosa and Fellous, 1984). In all cases examined, increased cell surface expression of class I antigens was correlated with increased transcript levels.

DeMars and co-workers have recently isolated mutants from the B-lymphoblastoid cell line, LCL 721, which do not express class I antigens at the cell surface. These mutants were obtained by exposure of cells to mutagenic doses of γ-irradiation followed by treatment with complement and antibodies specific for HLA class I or class II molecules on LCL 721 (Kavathas *et al.*, 1980). Variant clones were isolated with a frequency of $\sim 4 \times 10^{-5}$. A large number of mutants have now been generated and characterized by karyotyping and molecular genotyping. Clones which lost the structural gene encoding the selected marker were the most common type of mutant. However, some clones retained the gene encoding the selected marker, yet did not express the protein at the cell surface. We have used one of these mutants, called .174, to study class I antigen regulation (Salter *et al.*, 1985).

The mutant .174 was derived by a two-step selection procedure. LCL 721 was γ-irradiated and exposed to complement and alloantiserum specific for HLA-B8. The resulting clone, called .45, has a large deletion encompassing the entire HLA region of one copy of chromosome six (Orr and DeMars, 1983). .45 was re-irradiated and treated with a class II antigen-specific monoclonal antibody and complement to yield .174. This clone expresses no class II antigens and contains no genes coding for functional class II molecules (DeMars, 1984). Expression of class I antigens was also affected. The B5 antigen cannot be detected serologically on .174, and A2 antigen expression is reduced by 70–80% compared with the .45 parental cell line (DeMars *et al.*, 1984). Both the A2 and B5 genes are still present in .174, however, and can be re-expressed upon fusion with wild-type B-LCL (DeMars *et al.*, 1985) or T-LCL (Salter *et al.*, 1985).

We produced a hybrid of .174 and the T-LCL CEM^R, called 174XCEM.T1, which expresses high levels of the A2 and B5 antigens as well as high levels of CEM^R-encoded class I antigens (Salter *et al.*, 1985). A variant of the hybrid called 174XCEM.T2 was isolated which has lost both CEM^R-derived copies of chromosome six, and concomitantly reverted to low class I antigen expression phenotypically identical to .174. Thus we concluded that a *trans*-acting element necessary for high levels of

class I antigen expression is encoded on chromosome six. This gene is presumably located within the region of known homozygous deletions of .174. In the present study, we have followed the synthesis of the A2 and B5 antigens in .45 and 174XCEM.T2. These cells provide a unique opportunity for studying regulation of class I antigen expression, and may be useful in understanding biosynthetic pathways utilized by other membrane proteins.

Results

Transcription of HLA-A2 and B5 genes in 174XCEM.T1 and 174XCEM.T2

By Northern blot analysis, DeMars and co-workers have shown that .174 synthesizes HLA-A and -B locus mRNA (DeMars *et al.*, 1985). We therefore compared the levels of HLA-A and -B locus transcripts in the hybrid 174XCEM.T1 and the low class I antigen-expressing variant 174XCEM.T2. Figure 1 shows that both cell lines contain abundant amounts of HLA-A and -B locus mRNA. Therefore, the defect in 174XCEM.T2 does not prevent transcription of class I genes and must affect a later biosynthetic process.

2-D gel electrophoretic analysis of class I antigens from .45 and 174XCEM.T2

Detergent lysates of .45 and 174XCEM.T2 cells labeled for 4 h with [^{35}S]methionine were prepared and used for immune precipitation studies with the monoclonal antibodies w6/32 and BB7.2. w6/32 recognizes a monomorphic determinant present on all HLA-A, -B and -C heavy chains when associated with β_2m (Brodsky and Parham, 1982). The antibody does not react with free heavy chains. Likewise, BB7.2 recognizes a determinant present only on the A2 molecule. We used .45 instead of 174XCEM.T1 for this study because the A2 and B5 antigens only are present in .45, whereas 2-D gels of 174XCEM.T1 are complicated by additional spots corresponding to CEM^R-derived antigens. As shown in Figure 2, w6/32 precipitates three sets of spots visible on 2-D gels from the .45 lysate. The most acidic of these are actin contaminants which are also present in the non-specific control precipitation. The most basic series of spots are also precipitated by BB7.2 and are therefore A2-related molecules. Directly below actin is a series of spots which are presumably HLA-B5 related. Although it remains a formal possibilty that these are HLA-C related, it seems unlikely since C locus antigens comprise only a small amount of total cellular class I protein. Precipitation from .45 with a monoclonal antibody specific for the B5 molecule has not been successful. Precipitation from 174XCEM.T2 gives a different pattern. No spots corresponding to the putative B5 molecules of .45 are evident on the gel. Overexposure of the film (not shown) shows very faint spots in the region below actin. In addition, the A2-related spots are fainter in 174XCEM.T2, especially in the BB7.2 precipitate. These experiments indicate that the cell surface class I phenotype of 174XCEM.T2 correlates with the levels of β_2m-associated A2 and B5 heavy chains.

Total levels of heavy chains in .45 and 174XCEM.T2

A rabbit antiserum called serum F which binds heavy chains with or without associated β_2m was used to measure total heavy chain levels in cells labeled for 4 h. Figure 3 shows 2-D gel analysis of .45 and 174XCEM.T2 lysates precipitated with serum F or with normal rabbit serum. Serum F specifically precipitates class I heavy chains from both cell lines. Alignment of the two autoradiograms using actin as a marker indicates that two sets of spots are precipitated from .45 and 174XCEM.T2, but that only the more basic species (indicated by arrows in Figure 3) are precipitated from 174XCEM.T2. This is consistent with low levels of sialic acid addition in 174XCEM.T2. Comparison of Figures 2 and 3 indicates that HLA-A2 and B5 heavy chains are synthesized in 174XCEM.T2, but that the association of β_2m with heavy chains, and with B5 heavy chains in particular, is impaired.

Fig. 1. Northern blot analysis. Total cytoplasmic RNA from 174XCEM.T1 (**lane 1**), 174XCEM.T2 (**lane 2**) and the T-LCL, CEM^R (**lane 3**) was electrophoresed in 0.9% agarose–formaldehyde gels and transferred to nitrocellulose filters. Filters were hybridized to probes specific for HLA-A and HLA-B locus sequences. CEM^R RNA (**lane 3**) was included on gels as a control, since CEM^R synthesizes low amounts of class I mRNA (Howell *et al.*, 1984).

Sequential precipitations with β_2m-specific serum and serum F

To determine the extent of heavy chain association with β_2m in .45 and 174XCEM.T2, lysates were precipitated twice with an excess of rabbit anti-human β_2m serum and StA to remove β_2m-associated HLA heavy chains. Remaining heavy chains in the lysate were then precipitated with serum F and samples were analyzed by SDS-PAGE. Lanes 1 and 3 in Figure 4 show equal amounts of .45 lysate precipitated with serum F following precipitation with anti-β_2m and normal rabbit serum, respectively. Density scanning of the autoradiogram indicates that the amount of heavy chain is reduced by 80% following precipitation by anti-β_2m serum and that 80% of the heavy chains in .45 are therefore β_2m associated. In contrast, 174XCEM.T2 heavy chains are largely unassociated, as the amount of heavy chain in the lysate is reduced by $<20\%$ by pre-precipitation with anti-β_2m serum (lanes 4 and 6, Figure 4).

Kinetics of class I antigen assembly and glycosylation in .45 and 174XCEM.T2

In pulse–chase experiments using 174XCEM.T2, when w6/32 was used as a precipitating antibody, little class I HLA antigen was detectable throughout a 60 min chase following a 5 min pulse with [^{35}S]methionine (data not shown). Thus, by this criterion, extensive association of class I heavy chains with β_2m does not occur even transiently in this cell line. In contrast, the kinetics of assembly in .45 were similar to those already published for

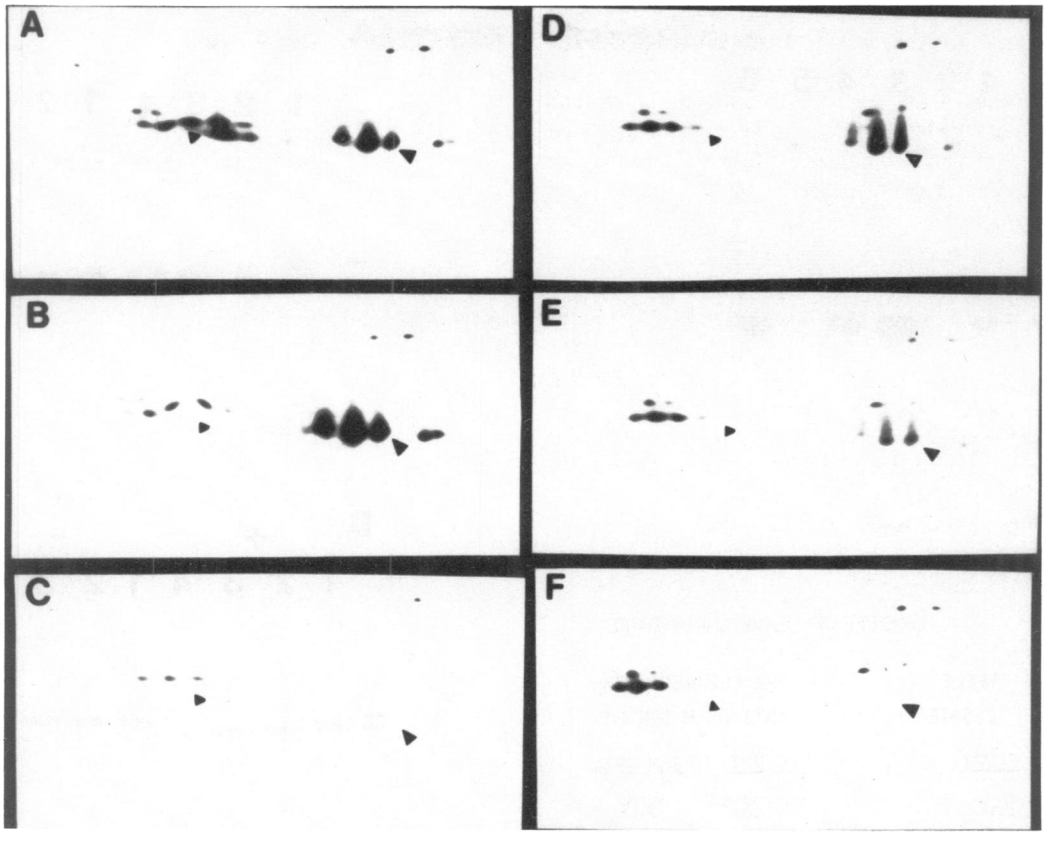


Fig. 2. Immune precipitations of β_2m-associated class I antigens. Cells were labeled for 4 h with [^{35}S]methionine. Class I antigens from .45 (**panels A–C**) and 174XCEM.T2 (**panels D–F**) were precipitated from [^{35}S]methionine lysates with w6/32 (**A** and **D**) and BB7.2 (**B** and **E**). GAP A3 (**C** and **F**) was included as a negative control antibody. Electrophoresis was from right to left (basic to acidic) in the first dimension, and from top to bottom in the second dimension 10.5% polyacrylamide gels. HLA-A2-related spots are marked by a large arrowhead and putative HLA-B5-related spots are marked with a small arrowhead.

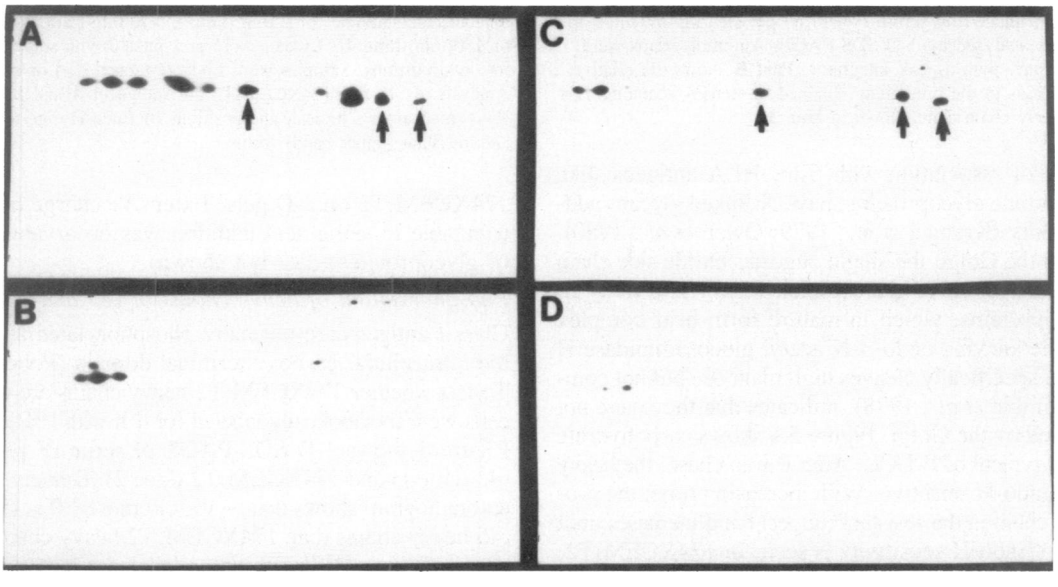
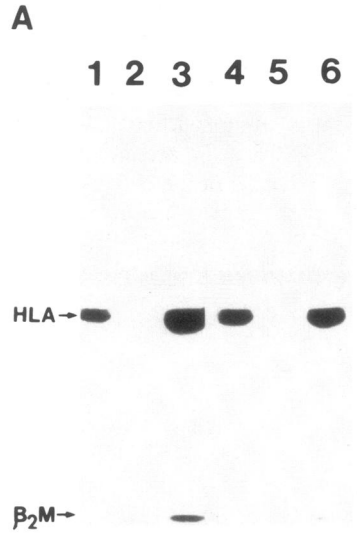


Fig. 3. Immune precipitations of total class I heavy chains. .45 cells (**panels A** and **B**) or 174XCEM.T2 (**panels C** and **D**) were labeled for 4 h with [^{35}S]methionine, and heavy chains were precipitated with serum F (**A** and **C**) or normal rabbit serum (**B** and **D**). 2-D gel analysis was performed as described in Figure 2.

other B-LCL (Krangel *et al.*, 1979; Owen *et al.*, 1980). Heavy chain association with β_2m was complete by 15–30 min. When pulse–chase lysates were precipitated by serum F and analysed by SDS-PAGE, class I heavy chains were present at similar intensities throughout the chase period for .45 and 174XCEM.T2 until 180 min, when a lessening of intensity was apparent in the case of 174XCEM.T2 (Figure 5B). This may indicate a more rapid turnover of the majority of heavy chains in this cell line



B

DENSITY OF HEAVY CHAIN BAND

	WHOLE LYSATE:	PRE-CLEARED WITH ANTI-β_2M SERUM:	
	F PPI.	F PPI.	B_2M PPI.
.45	1.00	0.28	0.00
174xCEM.T2	0.50	0.43	0.00

Fig. 4. Sequential immune precipitation with anti-β_2m serum and anti-heavy chain serum. .45 cells (**lanes 1–3**) and 174XCEM.T2 cells (**lanes 4–6**) were labeled for 4 h with [^{35}S]methionine. Lysates were pre-precipitated twice with anti-β_2m serum, then precipitated with serum F (**lanes 1** and **4**) or anti-β_2m serum (**lanes 2** and **5**) of **part A. Lanes 3** and **6** show equal amounts of lysate precipitated by serum F with no pre-clearing by anti-β_2m serum. Samples were analyzed by 1-D SDS-PAGE. Autoradiograms were scanned by laser scanner with a peak integrator. **Part B** shows the relative amounts of heavy chains in the precipitate obtained by density scanning as a proportion of the heavy chain band shown in **lane 3**.

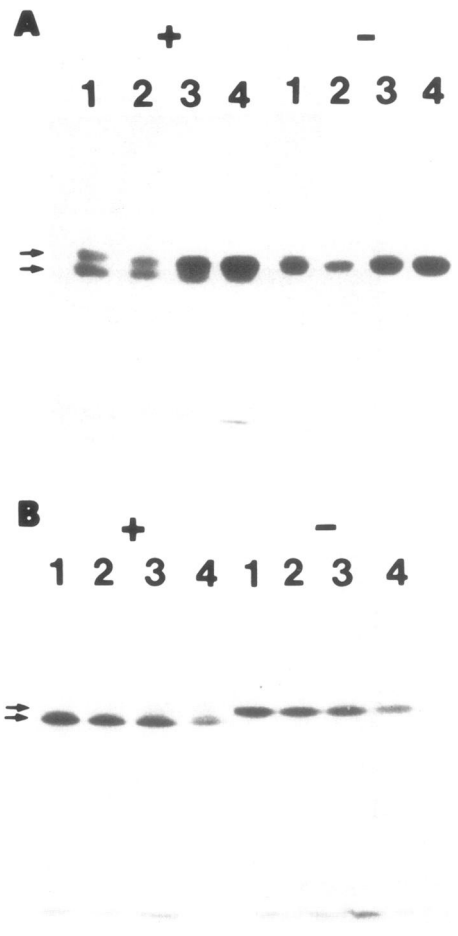


Fig. 5. Endo H treatment of class I heavy chains. Cells (15×10^6) were pulsed for 5 min with [^{35}S]methionine (0.75 mCi), then chased with unlabeled methionine for 0 min (**lane 1**), 30 min (**lane 2**), 60 min (**lane 3**) or 180 min (**lane 4**). Lysates were precipitated with serum F and divided into two portions. Samples were Endo H treated (+) or mock treated (–). Analysis of .45 and 174XCEM.T2 are shown in **A** and **B**, respectively. Two small arrows indicate the positions of Endo H-digested (lower band) and -resistant (upper band) forms.

due to their lack of association with β_2m. HLA antigens, like many other membrane glycoproteins, have N-linked glycans added co-translationally (Krangel *et al.*, 1979; Owen *et al.*, 1980). After transport to the Golgi, the single oligosaccharide side chain is trimmed and converted to the complex form. Sensitivity of membrane glycoproteins, which in mature form bear complex side chains, to the enzyme endo-β-N-acetyl glucosaminidase H (Endo H), which specifically cleaves high mannose but not complex glycans (Trimble *et al.*, 1978), indicates that they have not yet been processed by the Golgi. Figure 5A shows carbohydrate processing in .45 typical of B-LCL. After 0 min chase, the heavy chain is largely Endo H sensitive. With increasing time, the proportion of heavy chain in the resistant (upper) band increases until 60 min, when no Endo H sensitivity is seen. In 174XCEM.T2, heavy chains remain Endo H sensitive for at least 3 h after synthesis, as shown in Figure 5B. We conclude that most of class I molecules are not transported and processed normally in this mutant. The mobility differences observed between the class I heavy chains in Figure 3 presumably reflect sialic acid addition to the complex N-linked oligosaccharide of HLA heavy chains in .45, and no addition to the high mannose carbohydrate of those in 174XCEM.T2. A general defect in carbohydrate processing was ruled out by analysis of total cellular glycoproteins for 174XCEM.T2 on 2-D gels. Extensive charge heterogeneity attributable to sialic acid addition was observable for a number of glycoproteins (data not shown).

Phosphorylation of heavy chains of .45 and 174XCEM.T2

Class I antigens are normally phosphorylated at a single site in the intracellular carboxy-terminal domain (Pober *et al.*, 1978). To test whether 174XCEM.T2 heavy chains are phosphorylated, cells were metabolically labeled for 4 h with [^{32}P]orthophosphate. Figure 6 shows 1-D SDS-PAGE of serum F precipitates from .45 (lane 1) and 174XCEM.T2 (lane 2). Density scanning of the autoradiogram shows that ~10-fold more ^{32}P is incorporated into .45 heavy chains than 174XCEM.T2 heavy chains. Scanning of corresponding [^{35}S]methionine-labeled bands (Figure 4) shows only 2-fold less material in 174XCEM.T2. Therefore, this difference in phosphate incorporation is not due solely to lower amounts of class I protein in the 174XCEM.T2 lysate. Also, ^{32}P incorporation into total protein from the two cell lines is not significantly different (Figure 6, lanes 3 and 4).

Structural features of 174XCEM.T2 class I antigens expressed at the cell surface

As mentioned earlier, 174XCEM.T2 expresses a residual amount

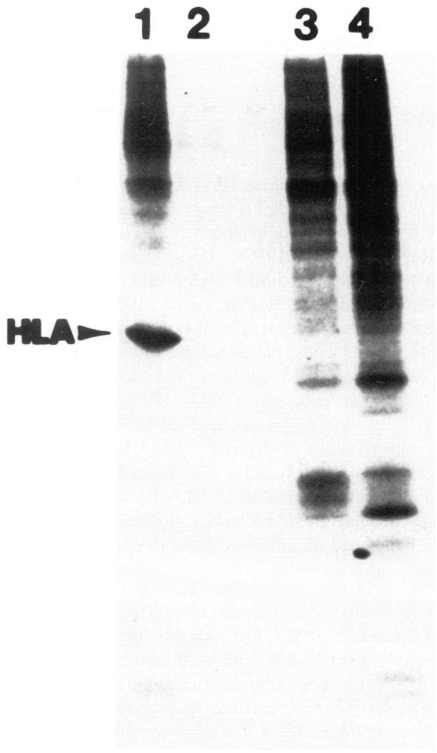


Fig. 6. Phosphorylation of class I heavy chains. Cells were labeled with [^{32}P]orthophosphate for 4 h, and serum F-precipitated heavy chains analyzed by 1-D SDS-PAGE. Heavy chains from .45 (**lanes 1** and **3**) and 174XCEM.T2 (**lanes 2** and **4**) were precipitated with serum F (**lanes 1** and **2**). **Lanes 3** and **4** show acetone-precipitated material from whole cell lysates.

Table I. Analysis of HLA class I antigen expression by flow cytometry

	Corrected mean fluorescence channel (MFC)[a]	
	w6/32	Serum F
.45	470	366
174XCEM.T2	104	64
Ratio of .45 to 174XCEM.T2 MFC	4.5	5.7

[a]Mean fluorescence channels were calculated on a linear scale and negative control antibody value subtracted from experimental values to give corrected mean fluorescence channel. Negative control antibody values were comparable for all four combinations of cells and antibodies and ranged from 20 to 37.

of A2 antigen at the cell surface. The antigen is bound by w6/32 and BB7.2 and is therefore β_2m associated (Salter *et al.*, 1985). Immunofluorescence studies with serum F were used to determine if only β_2m-associated class I molecules are found on the plasma membrane of 174XCEM.T2. If free heavy chains were also present on the surface of 174XCEM.T2, the ratio of serum F binding to w6/32 binding would be expected to be higher than is observed in normal class I antigen-positive cell lines. As shown in Table I, the ratios of serum F to w6/32 binding to .45 and 174XCEM.T2 are nearly identical. No free heavy chains are detectable on the surface of 174XCEM.T2 by this criterion.

To determine whether cell surface class I antigens are Endo H sensitive like the bulk of heavy chains from 174XCEM.T2, w6/32 was pre-bound to [^{35}S]methionine-labeled cells before detergent solubilization. Excess unlabeled cells were added during lysis to prevent binding of dissociated w6/32 to labeled intracellular heavy chains. StA precipitates were treated with Endo H, then analyzed by 1-D SDS-PAGE. Heavy chains from the surface of both .45 and 174XCEM.T2 proved to be Endo H resistant (data not shown). From these results, we conclude that class I antigens on the surface of 174XCEM.T2 are structurally distinct from the majority of heavy chains in the cell. Association with β_2m may be required for carbohydrate processing of the heavy chain and for transport to the cell surface. Carbohydrate addition *per se* appears not to be required for transport, as tunicamycin treatment, which inhibits N-linked glycosylation, does not stop transport of class I dimers to the cell surface (Owen *et al.*, 1979; Ploegh *et al.*, 1979).

Discussion

HLA class I antigen biosynthesis has been studied intensively in several B-LCL (Krangel *et al.*, 1979; Owen *et al.*, 1980). Although the kinetics of processing and transport vary between different cell lines, the temporal sequence of events has been determined. The nascent polypeptide is core glycosylated in the lumen of the rough endoplasmic reticulum (RER). Heavy chains bearing high-mannose oligosaccharides associate with β_2m, presumably in the RER, 5–15 min after chain synthesis is complete. Processing of the carbohydrate to the complex form in the Golgi occurs ~20–40 min after synthesis and, beginning at 30–60 min, mature class I antigens appear at the cell surface.

The data presented in this paper suggests that 174XCEM.T2, which expresses reduced levels of HLA-A2 and no detectable HLA-B5 at the cell surface (Salter *et al.*, 1985), does so as a result of defective assembly of heavy chain–β_2m dimers. Electrophoretic analysis shows that heavy chain is synthesized and core glycosylated normally. However, the majority of heavy chains do not associate with β_2m as shown by sequential precipitation with anti-β_2m and anti-heavy chain sera (Figure 4). The single N-linked glycan of the heavy chain remains Endo H sensitive for at least 3 h after synthesis (Figure 5), consistent with failure of the heavy chain to traverse the Golgi. The majority of class I heavy chains do not appear at the cell surface. The small amounts of A2 antigen which are transported to the cell surface of 174XCEM.T2 are β_2m associated, shown by surface binding of w6/32 and BB7.2 (Salter *et al.*, 1985), and Endo H resistant, and thus appear to be processed properly. Failure to associate with β_2m apparently prevents further carbohydrate processing and transport of most heavy chains in 174XCEM.T2.

The requirement for β_2m association for surface expression of class I HLA antigens was originally suggested by analysis of the mutant B-LCL Daudi. β_2m is not synthesized in this cell because the initiation codon of the β_2m mRNA is defective (Rosa *et al.*, 1983). Class I heavy chains in Daudi, like those in 174XCEM.T2, are synthesized, remain Endo H sensitive, and are not transported to the cell surface (Ploegh *et al.*, 1979; Sege *et al.*, 1981). Somatic cell hybrids of Daudi and B-LCL which produce β_2m express class I antigens encoded by Daudi (Arce-Gomez *et al.*, 1978; Fellous *et al.*, 1977). Thus Daudi heavy chains are not aberrant and can be expressed on the plasma membrane in association with β_2m.

The class I phenotype of 174XCEM.T2 is superficially similar to that of Daudi. However, 174XCEM.T2 synthesizes abundant β_2m as measured by inhibition radioimmunoassay (Salter *et al.*, 1985). Several lines of evidence indicate that this β_2m is normal. First, β_2m from 174XCEM.T2 focuses identically on 2-D gels to that from other cell lines tested (unpublished results). Second, 174XCEM.T2 should contain multiple copies of the β_2m

gene encoded on chromosome 15. Karyotyping indicates that 174XCEM.T2 in fact has three copies of chromosome 15 (unpublished results). It is extremely unlikely that all of these genes code for mutant molecules which can be recognized serologically, but are unable to combine with heavy chains. In addition, 174XCEM.T2 was selected from the class I-positive 174XCEM.T1 hybrid parent with a class II antigen-specific antibody and complement. No selective pressure against class I determinants or β_2m was therefore exerted to generate the phenotype of 174XCEM.T2. A third argument against lack of normal β_2m in this cell is that the low class I antigen phenotype is leaky. Residual A2 antigen at the cell surface is β_2m associated. We conclude that normal β_2m and normal heavy chains are synthesized in 174XCEM.T2, but are not efficiently assembled and transported to the cell surface. These results suggest that an additional molecule absent from 174XCEM.T2 is required for assembly of the class I dimer.

The mechanism by which the HLA-linked gene identified by these experiments and those of DeMars and co-workers (DeMars *et al.*, 1985) facilitates class I heavy chain–β_2m association remains a matter of speculation. One possible model is that the gene encodes an enzyme which enhances dimer formation. It has been shown in a number of systems that β_2m associated with class I heavy chains can spontaneously exchange with free β_2m in solution (Schmidt *et al.*, 1981; Bernabeu *et al.*, 1984). Thus an enzymatic model would require that newly synthesized class I heavy chains which have never been associated with β_2m require the enzyme for β_2m addition, while the exchange reaction exhibited by native heavy chain–β_2m dimers does not. One function of such an enzyme could be to facilitate a conformational change in the class I heavy chain required for β_2m addition. An equilibrium in the absence of enzyme favoring the inactive heavy chain conformation but with a small proportion in the active conformation might explain the β_2m association and cell surface expression of a proportion of the HLA-A2 molecules in 174XCEM.T2. The difference in levels of association between HLA-A2 and B5 may represent allelic or locus differences in such an equilibrium. Alternative mechanisms involving impaired transport of class I heavy chains and/or β_2m to a subcellular site where assembly occurs, e.g. the *cis* Golgi, cannot at present be ruled out.

Some evidence supporting a requirement for a molecule which facilitates class I heavy chain β_2m association come from cell-free translation studies (Ploegh *et al.*, 1979). Poly(A)$^+$ RNA from the B-LCL, JY, was translated in a rabbit reticulocyte lysate system, and the products were precipitated with w6/32, anti-heavy chain serum or anti-β_2m serum. Both β_2m and heavy chains were synthesized, but did not assemble even in the presence of dog pancreas microsomes, which cleave signal sequences from both subunits and should allow their insertion into closed membrane compartments. Lack of an assembly molecule necessary for formation of the dimer could explain this result, although it is not clear why such a molecule would not be translated from the poly(A)$^+$ RNA pool.

Post-translational modifications other than glycosylation of class I antigens are also affected in 174XCEM.T2. Phosphorylation of heavy chains appears to be inhibited 10-fold. In preliminary experiments we have found that the small amount of phosphorylated heavy chain precipitated from 174XCEM.T2 with serum F is Endo H resistant, and therefore probably found at the cell surface. From this result, we conclude that phosphorylation of class I antigens occurs after heavy chains associate with β_2m and become Endo H resistant probably in the Golgi or during transport to the plasma membrane.

Materials and methods

Cell lines

The B-LCL 721 expresses the antigens HLA-A1, -B8, -DR3, -A2, -B5 and -DR1 (Kavathas *et al.*, 1980). The single haplotype loss variant .45 was derived from LCL 721 after γ-irradiation and complement-mediated selection with a B8-specific alloantiserum (Kavanthas *et al.*, 1980). .45 expresses HLA-A2, -B5 and -DR1. Clone .174 was derived from .45 after γ-irradiation and complement-mediated selection against class II antigens (DeMars *et al.*, 1984). No serologically defined class II antigens are detectable on .174. It expresses reduced amounts of HLA-A2 relative to .45, and encodes, but does not express HLA-B5 (DeMars *et al.*, 1984). 174XCEM.T1 is a cloned hybrid of .174 and CEM^R which was produced as previously described (Salter *et al.*, 1985). 174XCEM.T2 is a variant derived from 174XCEM.T1 by complement-mediated selection against HLA-DR antigens. Both copies of chromosome 6 derived from CEM^R have been lost from 174XCEM.T2 (Salter *et al.*, 1985). Like .174, this cell line expresses low amounts of HLA-A2 and undetectable amounts of HLA-B5, although both the HLA-A2 and -B5 genes appear intact by Southern blot analysis (unpublished observation). Cell lines were maintained as previously described (Salter *et al.*, 1985).

Metabolic radiolabeling

Radiolabeling of cells with [^{35}S]methionine was performed as previously described (Machamer and Cresswell, 1982). For radiolabeling with [^{32}P]orthophosphate, 5×10^6 cells were washed 2 × in 2 ml of phosphate-free RPMI 1640 containing 3% fetal calf serum. After 60 min incubation at 37°C in phosphate-free medium, 2.5 mCi of [^{35}S]orthophosphate (carrier-free, Amersham) was added and the incubation continued for 4 h. Cells were washed 5 × in Dulbecco's phosphate-buffered saline (PBS). The cell pellet was lysed with detergent and lysate prepared as previously described (Machamer and Cresswell, 1982).

DNA probes

HLA-A and HLA-B probes were provided by Dr Harry Orr. Recombinant plasmid DNA was cut with restriction enzymes as described (Koller *et al.*, 1984) to generate A and B locus-specific probes.

Monoclonal antibodies

w6/32 (anti-HLA-A, B, C monomorphic) and BB7.2 (anti-HLA-A2) (Brodsky *et al.*, 1979) were provided by Drs Frances Brodsky and Peter Parham. GAP A3 (anti-HLA-A3) was produced in this laboratory (Berger *et al.*, 1982).

Antisera

Serum F is an antiserum produced in a rabbit as previously described (Cresswell *et al.*, 1981). It reacts with free or β_2m-associated HLA-A, -B and -C heavy chains and also with β_2m alone. A rabbit antiserum reactive with human β_2m was purchased from Miles–Yeda Laboratories in lyophilized form.

Northern blot analysis

2×10^8 cells were centrifuged, washed once in cold PBS then suspended in 7 ml of cold RSB (10 mM Tris, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$) containing vanadyl–ribonucleoside complexes (BRL). NP-40 was added to a final concentration of 1.5% and cells were vortexed. After 5 min on ice, nuclei were sedimented by centrifugation. 8 ml of RSB, 2 ml of 10 × TNE (10 × TNE is 100 mM Tris, pH 8.3, 1.5 M NaCl, 50 mM EDTA), 0.2 g SDS and 5 mg dextran sulfate were then added to the supernatant. The sample was phenol extracted twice, then once with chloroform containing 5% isoamyl alcohol, before ethanol precipitation. The RNA pellet was dissolved in 50 mM Tris pH 8.0, 1 mM EDTA. 10 μg of RNA (per lane) was then electrophoresed in 0.9% agarose–formaldehyde gels in running buffer (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA) for 300 V/h. The gel was soaked in 20 × SSC (20 × SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 1 h before blotting onto nitrocellulose filters (Schleicher and Schuell). Blots were hybridized in 6 × SSC containing 50% formamide (v/v), 12% dextran sulfate (w/v), 8 μg/ml poly(A), 8 μg/ml poly(C), 200 g/ml yeast type III RNA (Sigma), 9 μg/ml sonicated DNA from *Escherichia coli*, 50 μg/ml sonicated salmon sperm DNA, 0.01% each of bovine serum albumin, Ficoll and polyvinylpyrolidone, and 100 ng ^{32}P-labelelled DNA probe nick-translated to a specific activity of $\sim 3 \times 10^8$ c.p.m./μg. Filters were hybridized for 18 h at 42°C, then washed twice in 2 × SSC, 0.1% SDS for 15 min at room temperature and four times in 0.1 × SSC, 0.1% SDS for 20 min at 60°C before exposure to Kodak XAR-5 film.

Glycoprotein preparation

Glycoproteins binding *Lens culinaris* hemagglutinin were prepared as described (Machamer and Cresswell, 1982).

Immune precipitations

Precipitation of immune complexes with *Staphylococcus aureus* (StA) was performed as described (Machamer and Cresswell, 1982). In one experiment, a solid phase absorption technique was used to precipitate complexes as described (Cresswell, 1985).

Pre-binding of antibody to radiolabeled cells

Cells were radiolabeled with 0.5 mCi [^{35}S]methionine for 4 h as described (Machamer and Cresswell, 1982). 3 μg of purified w6/32 was added for 20 min at 4°C. Cells were washed 2 × in PBS, and a cell lysate was made as described (Machamer and Cresswell, 1982). 3 × 10^7 unlabeled cells were added to the radiolabeled cells before lysis. 250 μl of a 10% StA suspension was then used to precipitate antigen–antibody complexes. Pellets were washed 5 × with 0.05% Triton X-100 in 0.01 M Tris, 0.15 M NaCl, pH 7.4 before Endo H treatment and 1-D SDS-PAGE.

One-dimensional polyacrylamide gel electrophoresis

Electrophoresis was performed as described (Laemmli, 1970) on 7.5–15% linear gradient slab gels (SDS-PAGE).

Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional gels with isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension were run as described (Markert and Cresswell, 1982). A four to one ratio of 5/7 to 3/10 Biolyte ampholytes (Biorad) was used to obtain an IEF pH gradient of 4–7.5.

Endo H treatment

Endoglycosidase H treatment was performed as described (Machamer and Cresswell, 1984). Mock-digested samples were treated identically except that Endo H was not added.

Indirect immunofluorescence

Indirect immunofluorescence and analysis with an Ortho 50H Cytofluorograf were performed as described (Salter *et al.*, 1985). For analysis with w6/32, fluoresceinated goat anti-mouse immunoglobulin (Meloy) was used as a secondary reagent, and GAP A3 binding constituted the negative control. Normal rabbit serum was used as a negative control for serum F, and fluoresceinated *Staphylococcus aureus* protein A was the second reagent.

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