Peptide-induced Stabilization and Intracellular Localization of Empty HLA Class I Complexes

By Erik J. Baas, Hisse-Martien van Santen, Monique J. Kleijmeer,* Hans J. Geuze,* Peter J. Peters,* and Hidde L. Ploegh

*From the Department of Cellular Biochemistry, The Netherlands Cancer Institute, 1066 CX, Amsterdam; and the *Department of Cell Biology, Faculty of Medicine, University of Utrecht, Utrecht, The Netherlands*

Summary

The human cell line T2 has been reported to be class I assembly deficient, and accordingly expresses reduced amounts of HLA-A2 and no HLA-B5 at the cell surface. By immunoblotting we observe the steady-state class I heavy chain levels of T2 to be near normal when compared with the identical class I alleles of the wild-type cell line T1. In pulse chase experiments, formation of heavy chain β_2-microglobulin complexes is observed for both HLA-A2 and HLA-B5. Culture at reduced temperatures (26 or 20°C) does not increase the amount of class I molecules transported, unlike what has been reported for the class I assembly–deficient mouse mutant cell line RMA-S. The HLA-B5 and the HLA-A2 complexes formed by T2 are thermolabile in cell lysates, albeit to different degrees. The thermolability of HLA-B5 can be overcome by addition of HLA-B5-presentable peptides, obtained by trifluoroacetic acid extraction from an HLA-B5-positive cell line, underlining the necessity of peptide for class I stability and indicating that T2-derived class I complexes are devoid of peptide. Cytoplast fusion of T2 cells with RMA-S cells shows the defect in class I assembly of RMA-S to be similar to that of T2. Localization of class I molecules observed by immuno-electron microscopy reveals the accumulation in the T2 cell line of both HLA-B5 and HLA-A2 in the endoplasmic reticulum (ER). Class I molecules are present in all the cisternae of the Golgi complex of T2, but the ratio of HLA-A and -B locus products in the Golgi area differs significantly from that at the cell surface. We conclude that the requirement for peptide in transport of class I molecules manifests itself at a stage beyond the ER, most likely the Golgi area.

HLA class I molecules consist of an MHC-encoded heavy chain, the light chain β_2-microglobulin (β_2M),[1] and a third subunit, a peptide of 8–10 amino acids (1–3). It is this ternary complex that is seen by the TCR. Mutant cell lines with deficiencies in class I expression have been particularly useful in elucidating the assembly and intracellular transport pathways of MHC products.

The T2 cell line (4, 5) was produced by fusion of the B lymphoblastoid cell line .174, carrying a deletion in the MHC class II area, with the T cell line CEM. The fusion product (T1) was selected for loss of CEM-derived chromosome 6, resulting in the cell line T2. The T2 and .174 cell lines have very similar phenotypes, and have been described as being unable to properly assemble MHC class I molecules, based largely on the reduced cell surface expression of HLA-A2, and on the absence of HLA-B5 molecules at the cell surface. As peptides are now recognized to be the essential third subunit of class I molecules, the inability of T2 cells to engage in HLA-A2-restricted antigen presentation is in accordance with the suggestion that peptide loading of class I molecules is deficient in this cell line (6, 7). Biochemical analysis in support of the claim of impaired assembly has relied on the use of antibodies that react equally well with free class I heavy chains and properly assembled class I molecules (8–10).

Other mutants have been described that resemble T2 in their defect in class I assembly, notably the murine cell line RMA-S (11). It has been claimed for both T2 and RMA-S that addition of presentable peptides induces assembly of the class I molecules from the constituent subunits (12, 13). Heterodimeric class I molecules formed by RMA-S appear to be "empty" (14); they lack peptide and display a number of remarkable properties. They are far more potent in their ability to bind peptide than class I molecules obtained from normal cells. They also are thermolabile. When exposed to tempera-

[1] *Abbreviations used in this paper:* β_2M, β_2-microglobulin; endo H, endoglycosidase H, ER, endoplasmic reticulum.

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tures >26°C in detergent lysates, some epitopes associated with properly occupied class I molecules are lost, without destruction of the class I heavy chain itself, a phenomenon interpreted as partial unfolding of the $\alpha 1\alpha 2$ domain of the heavy chain. After exposure to elevated temperature, the ability to bind peptide is irreversibly lost. Peptide not only fulfills the role of the nominal antigen recognized by the T cell, but is also required for the proper structure or possibly even assembly of class I molecules.

Assembly of heavy chain, light chain, and peptide takes place relatively early in the biosynthetic pathway. Empty class I molecules are formed in the course of biosynthesis and are also present at the cell surface (15). Whether the normal route of assembly proceeds predominantly via a peptide–class I heavy chain intermediate, or via a class I–β_2M "empty" heterodimer (16), remains to be established. Cells that are unable to deposit class I molecules at the cell surface have been claimed to accumulate class I molecules in the endoplasmic reticulum (ER), but no direct cytochemical data in support of this statement are available (13, 17). In other cell lines with a deficiency in class I expression, the class I molecules have been reported to recycle between the ER and the *cis*-Golgi complex (18). We have investigated the early steps in biosynthesis of class I molecules in the T2 cell line, and conclude that initial assembly of the heavy chain and β_2M apparently does take place normally, but yields conformationally labile complexes that can be successfully stabilized by the addition of peptide. Here we have used class I locus-specific antibodies to determine the intracellular localization of the class I products in the T1 and T2 cell lines. The transport defect in T2 manifests itself at the level of the Golgi compartment, as well as by retention of class I molecules in the ER.

The defect causing impaired HLA class I assembly and transport in T2 has been mapped to a large deletion in the HLA class II region of the MHC. It is now known that several genes involved in HLA class I assembly are localized in this region, in particular, the genes encoding proteins with structural similarity to ATP-binding peptide transporters (TAP1 and TAP2, previously PSF1/2, HAM1/2 in humans and mice, respectively), but also genes encoding cytosolic proteasome components (19–23, reviewed in reference 24). Both types of proteins are likely to be involved in the provision of peptides to the ER-resident class I heavy chain and β_2M. We show that interspecific complementation of the defect in the T2 cell line can be achieved by fusion of T2 with RMA, but that the defect in T2 fails to be complemented by fusion with RMA-S, indicating functionally similar proteins to be affected in the latter two cell lines.

Materials and Methods

Cell Culture. Cell lines were cultured in DMEM (Gibco BRL, Gaithersburg, MD) containing 10% FCS, supplemented with 2 mM L-glutamine, and antibiotics. For cells cultured at 26°C or 20°C, 25 mM Hepes buffer (Flow Laboratories, Inc., McLean, VA), pH 7.4, was added to the medium.

Immunoprecipitation and Antibodies. The protocol used for immunoprecipitation and neuraminidase treatment of immunoprecipitates has been described in detail (25). In short, labeled cells are lysed in NP-40 containing lysis buffer (0.5% NP-40 [BDH Chemicals, Poole, England], 50 mM Tris, pH 7.4, 5 mM $MgCl_2$). Lysates are precleared by incubation with 2 μl normal mouse serum for 45 min, followed by addition of formalin-fixed *Staphylococcus aureus* in quantities sufficient for complete removal of antibody during the 30-min incubation. This is repeated once, to ensure complete removal of aspecific binding protein. Subsequently, precipitation with specific antibodies are performed. Precipitated material is washed four times before resuspension of the pellet in IEF sample buffer for analysis by IEF gel electrophoresis. All immunoprecipitations and washing of precipitated material are done on ice. Antibodies used are W6/32 (26), rabbit anti–free heavy chain serum (27), HC-10 and HC-A2 (28), anti-P53 (29), anti-galactosyl transferase (30). Neuraminidase (type VIII) was obtained from Sigma Chemical Co. (St. Louis, MO).

IEF. One-dimensional IEF gel electrophoresis was performed as described (25). Gels were fluorographed using DMSO/diphenyloxazole (PPO), and labeled proteins were detected by autoradiography to Kodak XAR-5 films.

Pulse-Chase Analysis. 24×10^6 cells were labeled for 5 min with 250 μCi [^{35}S]methionine, (sp act 1,200 Ci/mmol; Amersham Corp., Arlington Heights, IL), and after addition of 1 mM cold L-methionine, 3×10^6 cells per time point were harvested and lysed in NP-40 lysis mix. To the NP-40 lysis mix, 0.1 mM PMSF and 1 μg/ml trypsin inhibitor was added freshly in all experiments.

Class I Stability Assay. 16×10^6 cells were labeled with 250 μCi ^{35}S-methionine for 30 min in the presence of 5 μg/ml BFA (a kind gift of the Sandoz Co., Basel, Switzerland), preventing the acquisition of sialic acid residues by the heavy chain, facilitating the observation of differences in the amount of heavy chain recovered in these experiments. This procedure has no effect on stability as assayed here, as labeling in the absence of BFA gives equivalent results. After lysis in 800 μl NP-40 lysis mix, to which 10 mg/ml OVA (Sigma Chemical Co.) was added, 100 μl lysate, corresponding to 2×10^6 cells, was incubated for 1 h at the temperatures indicated. Immunoprecipitations from the lysate were performed as described.

TFA Extract Preparation and Class I Stabilization. TFA-extractable material was isolated from 10^8 cells by incubation with 4 ml 0.1% TFA, 0.9% NaCl for 30 min, at room temperature. Extracts were lyophilized and resuspended in water three times to remove TFA, and stored at −20°C, taken up in a final volume of 3 ml water. 11×10^6 T2 cells were labeled for 1 h with 250 μCi [^{35}S]methionine. After lysis in 1 ml NP-40 lysis mix, to lysate of 10^6 cells, TFA-extracted material in the amounts indicated and NP-40 lysis mix (total volume of 250 μl) was added. After a 30-min incubation on ice, mixtures were incubated for 1 h at 37°C, and immunoprecipitation and IEF analysis were performed as described.

Western Blot Analysis. 5×10^5 cells per lane resuspended in 40 μl IEF sample buffer were loaded and run on IEF. After IEF and Western blotting (31), the rabbit anti–heavy chain serum (dilution 1:3,000) was used as first antibody, and peroxidase-conjugated goat anti–rabbit Ig antibody (dilution 1:10^4; Sigma Chemical Co.) as second antibody. Chemiluminescence (RPN 2106 kit; Amersham Corp., Arlington Heights, IL) and exposure to film (Xomat-S, Kodak) was used to detect antibody reactivity.

Cytoplast Fusions. This protocol was adapted from reference 32. 9×10^6 T2 cells were labeled for 30 min with 250 μCi ^{35}S-methionine, and washed once with DMEM. 9×10^6 unlabeled fusion partner cells were mixed with 1.5×10^6 labeled T2 cells, mixed, and taken up in 3 ml DMEM, to which PHA (HA-16; Wellcome Diagnostics, Beckenham, UK) was added to a final concen-

tration of 50 μg/ml. Cells were transferred to 4-cm culture dishes (Falcon 3001; Becton Dickinson & Co., Mountain View, CA) coated with poly-L-lysine. Coating was done by incubation with 4 ml 10 μg/ml poly-L-lysine HBr, molecular weight 70,000–150,000 (Sigma Chemical Co.) in PBS for 1 h at room temperature, followed by repeated washing with water. Coated dishes were stored dry, at 4°C. Cells were incubated for 1 h at 37°C, and washed two times with DMEM without FCS. To the cells to be fused, 250 μl 50% (wt/vol) polyethylene glycol 1000 (BDH Chemicals, Poole, England) in PBS was added, and cells were fused for 1 min at 37°C. After fusion, cells were washed three times with DMEM free of FCS, and after addition of 3 ml DMEM containing 10% FCS, they were cultured for 3 h at 37°C. Cells were lysed in 1 ml NP-40 lysis mix, and precipitations were performed as described.

Immunoelectron Microscopy. Aliquots of T1 and T2 cell were mixed before fixation with 0.1 M phosphate buffer, 2% glutaraldehyde for 2 h at room temperature. Ultrathin cryosections and immunogold labeling were performed as described (33). Briefly, after fixation, cells were washed twice with 0.1 M phosphate buffer and twice with PBS, 0.15 M glycine. After embedding in 10% gelatin, at 4°C, small pieces of gelatin were infiltrated with 2.3 M sucrose, mounted, and frozen in liquid nitrogen. Ultrathin cryosections were immuno-double labeled with mAbs HC-10, HC-A2, P53, or rabbit anti-galactosyl transferase polyclonal serum. To visualize the antibodies, 15- and 10-nm gold particles bound to protein A were used. For visualization of mAbs HC-A2 and P53, rabbit anti-mouse IgG1 was used as a bridging antibody between the primary mAb and the protein A–gold complex. Neglectible low background gold was found without use of the first antibody. To determine the distribution of HC-10 and HC-A2 for T1 and T2, the number of gold particles representing HC-A2 and HC-10 were quantitated in double-labeled sections. Only gold particles within 20 nm of a membrane were counted. For each cell type, 26 cells were randomly selected and the gold particles for HC-10 and HC-A2 were counted directly in the microscope at an instrumental magnification of 10,000. To determine the ratio of HC-10 over HC-A2 in the Golgi complex, 35 Golgi areas were counted for both T1 and T2. The background labeling as determined for nucleus and mitochondria was 3%. Before mixing of T1 and T2 cells, we showed these to be cell populations homogeneous in class I labeling. T1 and T2 cells could therefore be distinguished unequivocally after mixing and processing of the samples.

Results

High Steady-State Levels of Class I Heavy Chains in T2. The total levels of class I heavy chains in T1 and T2 at steady state were assessed after separation of the subunits by IEF under denaturing conditions. Immunoblots were prepared using a rabbit–anti-class I heavy chain serum (27) and chemiluminescence for detection (Fig. 1). Densitometry of autoradiograms shows that the T2 cell line still contains relatively high levels of HLA-A2 and HLA-B5 heavy chains, estimated at ~50–70% of the total heavy chain level observed for T1. It is concluded that the HLA class I heavy chains present in T2 are rather stable and remain present for a prolonged time. For T1, the interpretation of the IEF pattern is complicated by the presence of sialylated HLA-B5 and the presence of the HLA-Aw30 and -Bw6 specificities, all showing considerable overlap in the focusing positions of their heavy chains. About 30% of the HLA-A2 heavy chains present in T2 carry

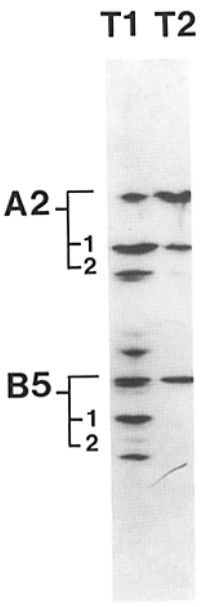

Figure 1. Steady-state HLA levels in T1 and T2. Total cell lysate of equal amounts of T1 and T2 cells was run on IEF, and, after immunoblotting, heavy chains were detected using the rabbit anti–heavy chain reagent. Focusing positions of unsialated and of the different sialylated forms of the HLA class I products are indicated. Note the complete absence of sialylated forms of HLA-B5 in T2.

sialic acids, whereas no sialated B5 heavy chains can be detected at steady-state levels in T2. The extent of sialylation observed for the HLA-A2 heavy chain at steady state is in good agreement with the levels of surface-expressed HLA-A2 detected by cytofluorimetry (4), and by immuno-electron microscopy (see below). Separation by IEF not only allows resolution of different allelic products, but also of their modifications, in particular the addition of sialic acids. Addition of sialic acids is reported to take place in the *trans*-Golgi complex and *trans*-Golgi network.

Proper Assembly of Class I Complex, but with Reduced Affinity for β_2M in the T2 Cell Line. Pulse-chase labeling experiments were performed on T1 and T2, and immunoprecipitates were prepared with the W6/32 antibody (Fig. 2). The reactivity of W6/32 has been carefully determined and is known to depend on proper association of class I heavy chain with β_2M (26).

The extent of intracellular transport of class I molecules may be judged from the acquisition of sialic acids by the heavy chains, resulting in a more acidic isoelectric point. In the T1 cell line, both HLA-A2 and HLA-B5 undergo this modification, which for both appears to be complete after 45 min of chase.

For the T2 cell line, expressing only HLA-A2 and HLA-B5, W6/32 clearly recovers a complex of heavy chain and β_2M, for both HLA-A2 and -B5. Note, however, the decrease in the quantity of β_2M recovered for T2, when compared with T1. Densitometry reveals that in the W6/32 immunoprecipitates from T2, approximately fourfold less β_2M is present than in those prepared from T1. This difference cannot be accounted for solely by the different amounts of class I products at steady state, which differ by no more than a factor of 2 (Fig. 1). Because W6/32 fails to react with free heavy chains, loss of β_2M from the immunoprecipitated class I molecules must have taken place in the course of the experiment, suggesting an intrinsic decrease in the stability of class

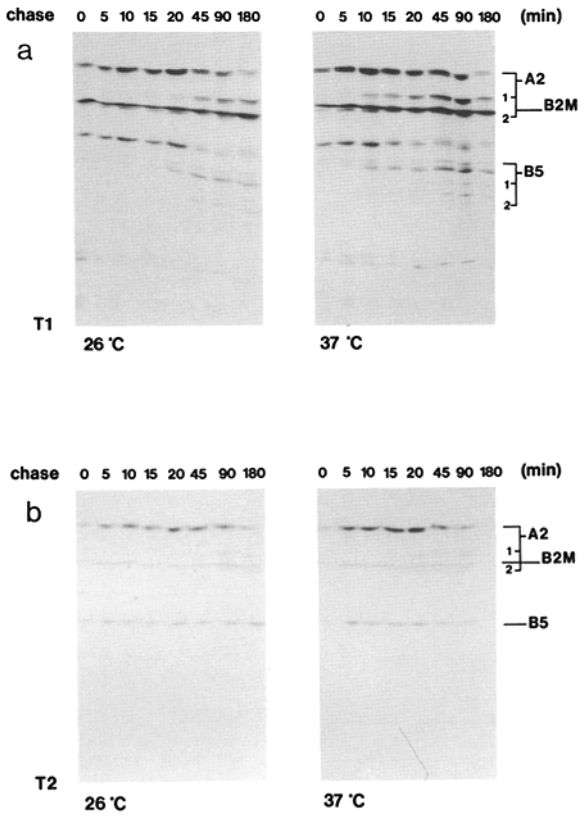

Figure 2. Pulse-chase analysis of W6/32-precipitated material from T1 (*a*) and T2 (*b*). Cells were pulse labeled for 5 min and chased for the times indicated, at either 26°C or 37°C. Immunoprecipitates were analyzed by IEF and detected by autoradiography. Focusing positions of the different sialylated forms of HLA-A2 and -B5 and of β_2M are indicated.

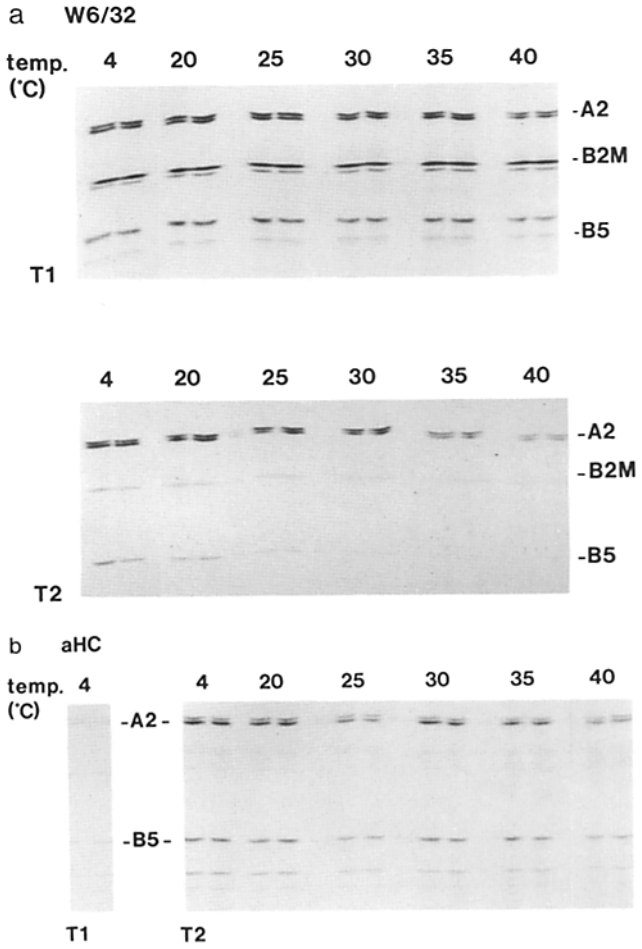

Figure 3. Stability of HLA molecules of T1 and T2 in cell lysates at different temperatures. (*a*) Material precipitated by W6/32 from T1 (*top*) and T2 (*bottom*) after incubation of the lysate for 1 h at the temperatures indicated. Analysis by IEF and detection by autoradiography was performed for all immunoprecipitates. IEF positions of the relevant subunits are indicated. (*b*) Free heavy chains precipitated subsequently from the same cell lysates.

I molecules of T2. Assembly is not impaired in a major way, because the relative amounts of HLA-A2 and -B5 heavy chains recovered by W6/32 for T2 show only very little reduction, when compared with T1. Whereas some sialylation is observed for HLA-A2, we have consistently been unable to observe this modification for the HLA-B5 complex (Figs. 1 and 2).

The Transport Defect of T2 Cannot Be Rescued by Reduced Temperature. Can the defect in transport of HLA class I molecules in the cell line T2 be rescued by reduced temperature, as was reported for the H-2K^b and D^b molecules in the mouse mutant cell line RMA-S? Pulse-chase experiments were therefore performed at reduced temperatures. When T2 cells were chased at 26°C, the rate, but not the extent of modification, was reduced for HLA-A2 (Fig. 2 *b*). We did not observe any sialylation of the HLA-B5 heavy chain at 26°C (Fig. 2 *b*), or at 20°C (not shown).

The class I molecules in T2 remain largely endoglycosidase H (endo H) sensitive (8, Baas et al.; data not shown). Densitometry revealed that at most 30% of the W6/32-reactive class I molecules acquired endo H resistance after prolonged chase (data not shown), again consistent with the level of HLA-A2 surface expression observed by cytofluorimetry (see also Fig. 1).

Thermolability of HLA-B5 and HLA-A2 in T2. The class I molecules produced by T2 have been reported to behave similarly, from a functional point of view, to those synthesized by the murine cell line RMA-S (12). In detergent lysates, empty murine class I molecules undergo an irreversible conformational change at 37°C, prevented by prior interaction with presentable peptides. The issue of stability of class I molecules was therefore explored further by exposing detergent lysates of labeled human T1 and T2 cells to increasing temperatures. For T1, lysates could be exposed to temperatures as high as 40°C without significant loss of W6/32-reactive material (Fig. 3 *a*). For the T2 cell line, the HLA-A2 molecules were rather resistant, and showed only slightly reduced recovery from lysates exposed to temperatures >35°C. However, the HLA-B5 molecules in T2 are clearly less stable than those in T1, also when compared with HLA-A2 in the

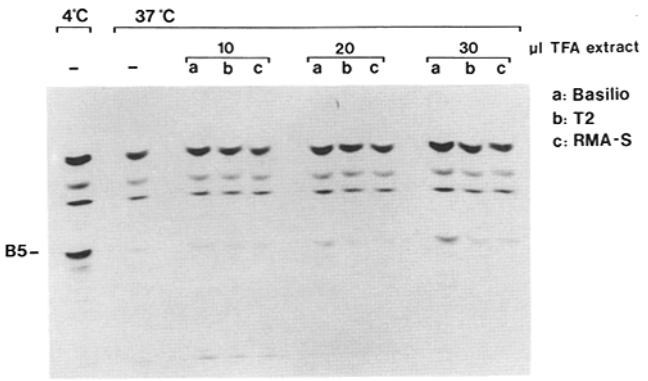

Figure 4. Stabilization of HLA-B5 molecules by the addition of peptide. Cell lysate of 10^6 metabolically labeled T2 cells was incubated with the indicated amounts of TFA-extracted material for 30 min before exposure for 1 h to 37°C or 4°C. The W6/32 immunoprecipitates were analyzed by IEF and detected by autoradiography. The focusing position of the HLA-B5 chain is indicated.

T2 cell line. The absence of class I complexes from T2 cell lysates exposed to elevated temperatures is not accompanied by increased breakdown of heavy chains. Free heavy chains, known to be rather susceptible to proteolysis, were recovered in near constant amounts from the lysate regardless of temperature of exposure (Fig. 3 *b*). The net loss observed for class I molecules therefore entirely applies to W6/32-reactive material.

Stabilization of HLA-B5 by TFA-extracted Naturally Processed Peptides. Since no HLA-B5-presentable peptides have been identified to date, we extracted the naturally processed peptides from properly folded HLA-B5 molecules with TFA (34), to assess the stabilizing capacity of HLA-B5-presentable peptides. Lysates from the T2 cell line were exposed at 37°C, in the absence or presence of increasing amounts of TFA-extracted material from the HLA-B5 homozygous cell line Basilio, to TFA extracts from the T2 cell line itself, and from RMA-S (Fig. 4). Only TFA extracts from Basilio were capable of conferring thermostability, and did so in a dose-dependent fashion. We thus suggest that the HLA-B5 molecules produced by T2 are indeed largely empty, as deduced by their ability to be protected from thermal unfolding by the inclusion of proper peptides, present in TFA extracts. Moreover, these HLA-B5 molecules fail to be transported to the compartment where sialyl transferases act, presumed to be the *trans*-Golgi complex or the *trans*-Golgi reticulum.

Subcellular Localization of Class I Molecules Analyzed by Immuno-electron Microscopy. The intracellular distribution of class I molecules was analyzed by immuno-electron microscopy. We have developed two mAbs, HC-10, and HC-A2, both of which were raised against purified free heavy chains of HLA-B and -A locus products, respectively (28). In immunoblots, these reagents show strong preference for the products of the locus against which the antibody was raised. Both HC-10 and HC-A2 react well on ultrathin cryosections, and in this sense are unique reagents. The widely different levels of plasma membrane labeling of class I molecules for T1 and T2 cells observed allowed their simultaneous fixation and further processing as a mixture of cells, while permitting unambiguous identification of each cell type. Thus, conditions for fixation, sectioning, and labeling were identical for both cell types. For the T1 cell line, HC-A2 and HC-10 reactivity was observed throughout the biosynthetic pathway, and on the cell surface. The reduction in labeling of the plasma membrane of T2 with HC-A2 is in good agreement with cytofluorimetry data, and also with the results from the pulse chase analysis (Fig. 5 *a*). The ratio of total plasma membrane labeling for T2 relative to T1 is 7% (HC-10) and 23% (HC-A2). For T2, the cell surface labeling observed with HC-10 is likely to be due to crossreactivity with HLA-A2 (28). If B5 were indeed completely absent from the cell surface of T2, this level of crossreactivity cannot exceed 20%.

Quantitation of ER-localized labeling (Fig. 5, *B* and *C*; Table 1) shows a sixfold relative increase in both HC-10 and HC-A2 labeling for T2 when compared with T1. Accumulation thus clearly takes place in the ER of T2 for both alleles.

In the Golgi area, labeling in all Golgi cisternae is observed in the T2 cell line for both HC-10 and HC-A2 (Fig. 6, *A* and *B*). Using P53 as a marker for the *cis*-Golgi complex (29), and galactosyl transferase as a marker for *trans*-Golgi localization (30), (Fig. 6, *C* and *D*), we note that HC-10 and HC-A2 reactivity is present throughout the Golgi complex of T2, including the *trans*-Golgi area. The ratio of HC-10- to HC-A2-reactive material in the Golgi complex is similar to T1 and T2 (1.3), whereas this ratio differs strongly for the plasma membrane. We thus conclude that the transport defect in T2 manifests itself not only in the ER, but also in the Golgi area.

The Class I Assembly-deficient Mouse Cell Line RMA-S Fails to Rescue the T2 Phenotype in Cytoplast Fusions. For T2, the exact mutation that leads to the failure to assemble class I molecules is known to include at least the deletion of a presumptive peptide transporter (19, 35), and in addition, an MHC-encoded subunit of a proteasome is lacking. To establish whether the defect in T2 can be complemented by RMA-S, cytoplast fusions of T2 cells with RMA-S cells were performed (Fig. 7).

T2 cells were labeled with [^{35}S]methionine, and fused with a large excess of unlabeled cells, for which T1, T2, RMA, and RMA-S were used. Only when T2 was fused with RMA or T1 did we observe sialylation of HLA-B5, indicative of transport of HLA-B5. Interspecific complementation of the transport defect in the T2 cell line can thus be achieved. The decreased recovery by W6/32 of HLA-B5 upon fusion or mock fusion with the murine cell lines (Fig. 7 *a*) can likely be attributed to the loss of human β_2M from the HLA-B5 molecules in the course of the experiment due to their intrinsic instability, and subsequent association thereof with murine class I heavy chains. Increased recovery of labeled β_2M after fusion or mock fusion with T1 or with T2 (Fig. 7 *b*) may be attributed to binding of labeled, T2-derived β_2M to cold heavy chains present in excess in the lysate. The defect in T2 cannot be complemented by RMA-S.

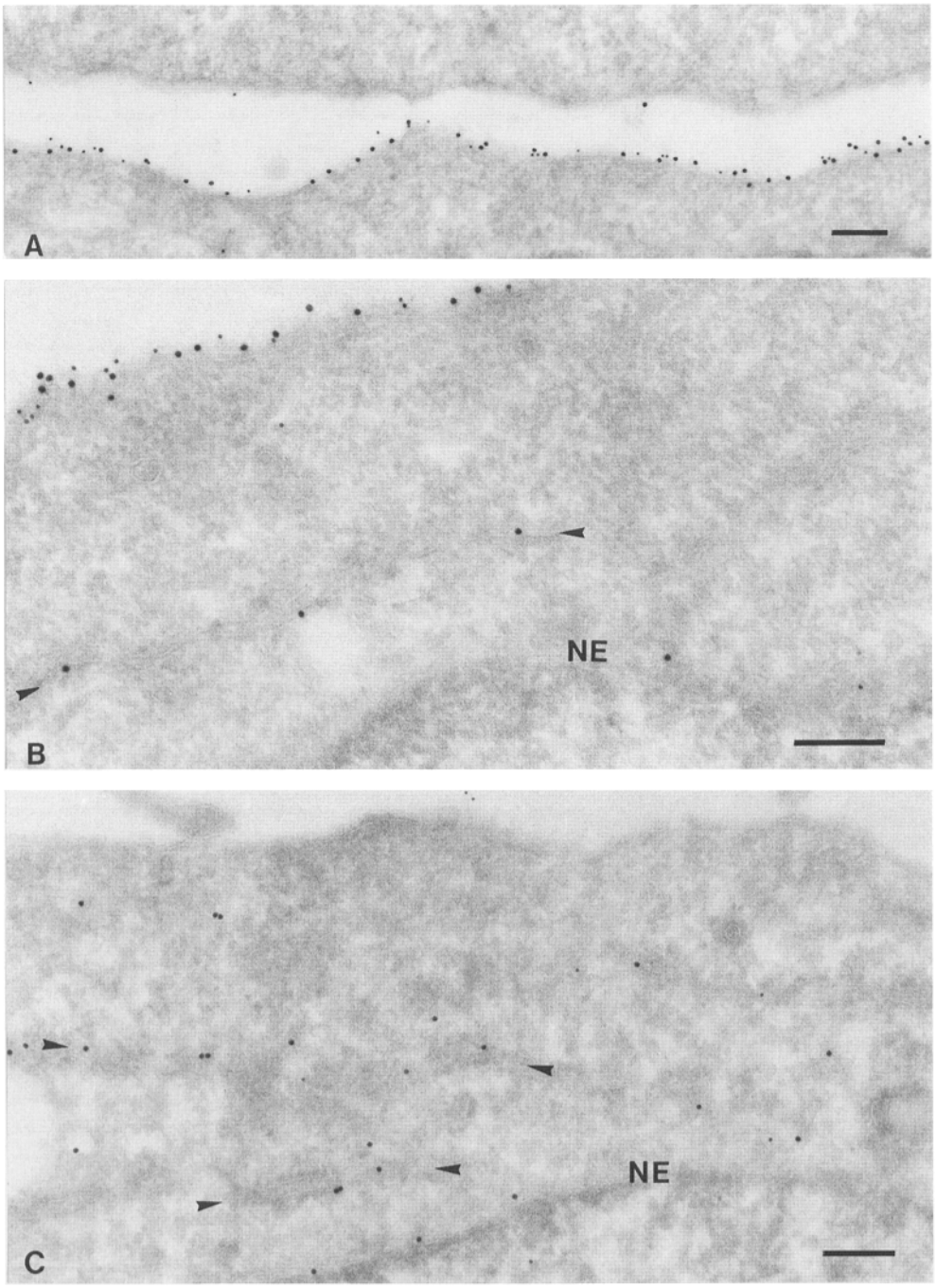

Figure 5. Immunogold detection of HC-A2 (10 nm gold) and HC-10 (15 nm gold). Bar: 0.1 μm. (*A*) Peripheral parts of T1 (*bottom*) and T2 (*top*) cells, showing the low cell surface expression of class I on T2 as compared with T1. (*B*) T1 cells with abundant labeling at the plasma membrane, but only little HC-A2 and HC-10 labeling in the ER (between *arrowheads*) and nuclear envelope (*NE*). (*C*) T2 cells showing heavier labeling of the ER (between *arrowheads*) and nuclear envelope when compared with T1 (*B*).

The reverse experiment, fusion of pulse-labeled RMA-S with unlabeled human cells, gave equivocal results, presumably because a transient reduction in temperature, difficult to avoid in the course of the fusion experiment, results in appreciable modification and transport of the mouse class I molecules (see reference 17; data not shown).

Discussion

The T2 cell line has been reported to be deficient in assembly and intracellular transport of HLA class I molecules. Not all class I alleles are affected equally: a low level of HLA-A2, but no HLA-B5, is detected at the cell surface (4, 5).

The early stages of biosynthesis of HLA class I molecules were studied in the T2 cell line by pulse-chase analysis. The epitope recognized by the W6/32 antibody is known to be critically dependent on the presence of β_2M, and W6/32-reactive class I molecules therefore contain β_2M. Properly assembled W6/32-reactive complexes of both HLA-A2 and HLA-B5 are formed in the T2 cell line at a rate and extent similar, if not identical, to those observed for T1. These complexes remain present for a prolonged time, as is the case for

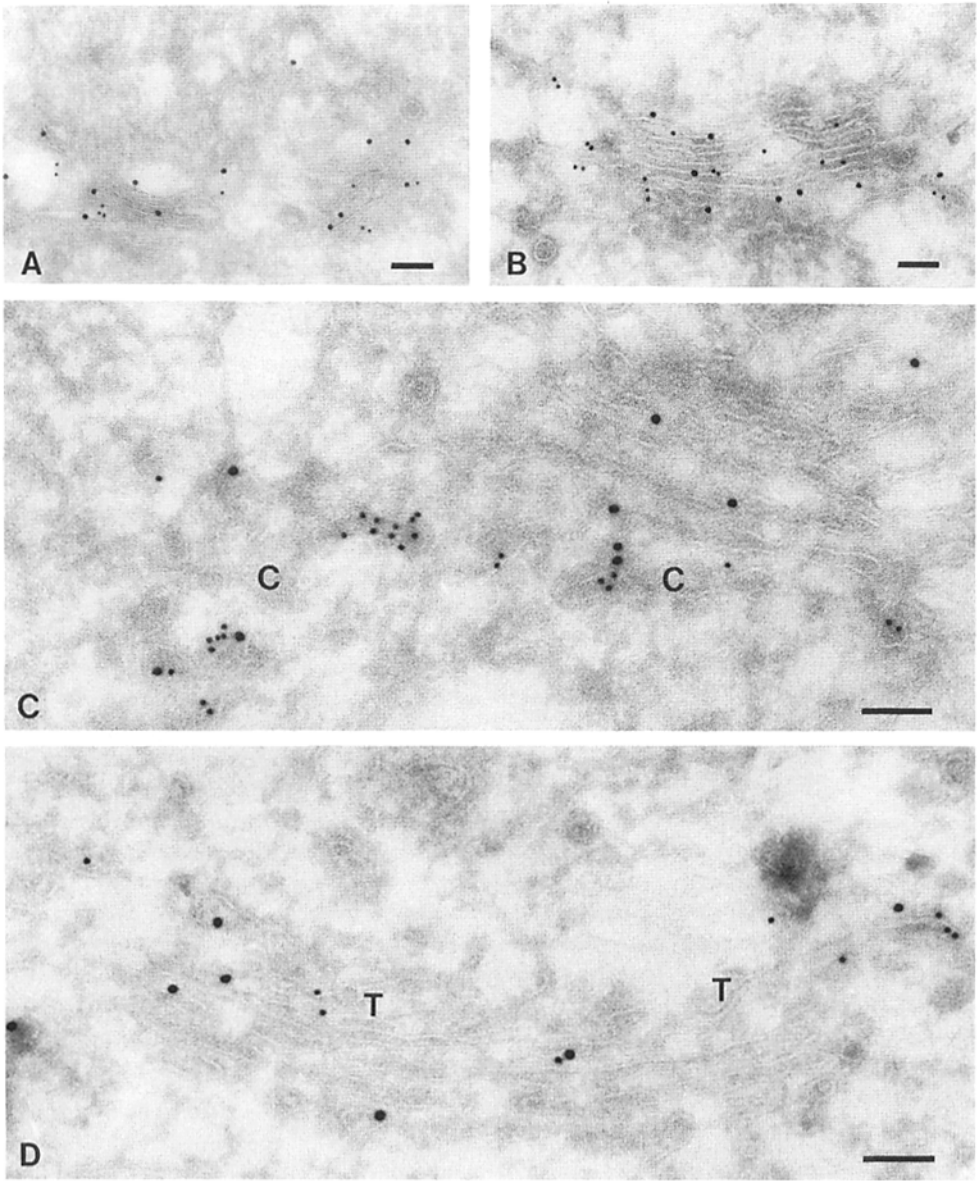

Figure 6. (*A* and *B*) Immunogold detection of HC-A2 (10 nm gold) and HC-10 (15 nm gold) in the Golgi areas of T1 (*A*) and T2 (*B*). Bar: 0.2 μm. (*C* and *D*) Golgi distribution in T2 of HC-10 (15 nm gold) and *cis*-Golgi marker P53 (10 nm gold; (*C*) and antigalactosyl transferase (10 nm gold; *D*) is shown. HC-10 labeling can be seen in all stacks of the Golgi area. Bar: 0.2 μm. (C) *cis*-Golgi; (*T*) *trans*-Golgi.

Table 1. *Distribution of the Immuno-gold Labeling for HC-10 and HC-A2 of T1 and T2*

	T1		T2	
	HC-10	HC-A2	HC-10	HC-A2
Plasma membrane	83	91	16	70
ER	10	3	69	20
Golgi	4	2	6	2
Endosomes/lysosomes	3	4	9	8

Numbers are percentage of total label observed for the indicated membrane fractions.

the HLA complexes formed by the wild-type cell line T1. Transport of the HLA-B5 complex to the *trans*-Golgi complex, or *trans*-Golgi reticulum, is not detectable in T2, as judged by the failure of the HLA-B5 heavy chain to acquire sialic acids. The level of sialylation of HLA-A2 seen in pulse-chase experiments indeed matches the level of surface expressed HLA-A2 as observed by cytofluorimetry, as well as by analysis of steady-state levels of HLA-A2. A decrease in the temperature of culture does not improve transport to the cell surface of either HLA-A2 or HLA-B5, unlike the case for class I products of the murine mutant cell line RMA-S (11). If the defect of T2 resembles that of RMA-S, as has been suggested (13), the lack of transport of HLA class I molecules at reduced temperature reflects either a property unique to human class I molecules, or it may be an indication that

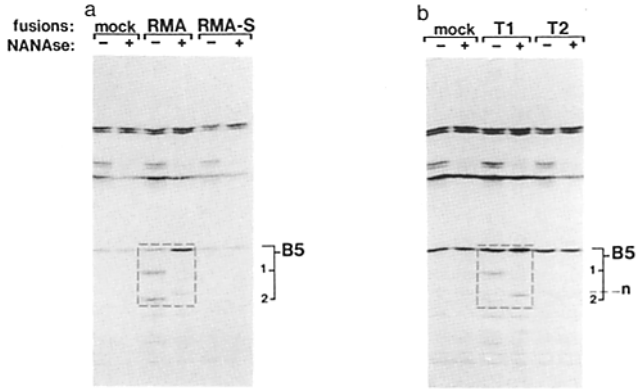

Figure 7. Cytoplast fusion experiments. After fusion, W6/32-reactive material was analyzed by IEF and autoradiography. Half of the immunoprecipitated material was first treated with neuraminidase, the other half was mock treated. (*a*) The left lanes show immunoprecipitated class I complex after mock fusion of labeled T2 cells with RMA cells, the middle lanes show this after fusion with RMA cells, and in the right lanes the result after fusion with RMA-S cells is shown. Boxed areas indicate the position where sialylated HLA-B5 molecules are detected. (*b*) W6/32 precipitations after fusion of labeled T2 cells with T1, T2, and mock fusion with T1 cells, respectively. The different forms of HLA-B5 are indicated on the right, and an unidentified heavy chain product, possibly a class I C-locus product (*n*).

the quality control mechanisms that preclude efficient surface deposition of class I molecules devoid of peptide are different for human and mouse cells. The results from cell fusion experiments performed between RMA-S and T2 further suggests the defect to involve homologous functions if not the identical genes. While this manuscript was in preparation, the transfection of RMA-S with TAP2 (HAM2 or mtp2) was reported (35–37). This transfection rescues, at least in part, surface expression of class I molecules. The inability to restore transport of class I molecules in the T2 cell line by fusion with RMA-S cytoplasts is thus most likely due to the fact that both cell lines share the lack of (functional) TAP-2 protein. In addition, the T2 cell line has been shown to lack the TAP1 (PSF1) subunit of the presumed peptide transporter complex (35), as well as an MHC-encoded proteasome subunit (23).

HLA-A2 is remarkable in its capacity to be expressed at the cell surface under conditions where other HLA class I alleles are retained intracellularly (9, 10; Fig. 2). This may reflect the capacity of the HLA-A2 molecule to be loaded with peptides of a different intracellular source (38).

In the course of the experiment, β_2M is lost from W6/32 immunoprecipitates prepared from T2 but not from T1, and the stability of class I complexes formed by T2 is apparently decreased. The reduced stability of the class I complexes synthesized by T2 is also reflected by the behavior of the HLA-A2 and HLA-B5 class I complexes of T2 in cell lysates. The HLA-B5 complex is unstable at temperatures >25°C, while incubation >35°C results in only a slight decrease in the amount of W6/32-reactive material for HLA-A2. We favor the notion that empty class I molecules partially unfold, but do not necessarily dissociate at elevated temperatures, as no increase of free heavy chains occurs where a reduction in the W6/32-reactive complexes is observed. Addition to the T2 cell lysate of naturally processed peptides, isolated by TFA extraction from a HLA-B5-expressing cell line, prevents disappearance of the W6/32 epitope on the HLA-B5 complex when exposed to elevated temperatures. We conclude that absence of peptide from the HLA-B5 complex in T2 contributes to the observed thermolability.

The use of anti-HLA heavy chain–specific antibodies with strong locus preference in their reactivity in immuno-electron microscopy has allowed us to define for the first time the intracellular localization of HLA class I molecules in T2 at the ultrastructural level. Quantitation of plasma membrane labeling for T1 and T2, shows that the cell surface levels of HLA-A2 and -B5 are determined accurately by this method. The intracellular localization of both HLA-A2 and HLA-B5 is affected in T2: a sixfold accumulation of both alleles is observed in the ER of T2 relative to T1. Double labeling for the *cis*- and *trans*-Golgi markers P53 and galactosyl transferase, respectively, shows that both HLA-A2 and HLA-B5 can still be transported into the Golgi area. In the Golgi complex, the ratio of HLA-A2 to HLA-B5 is markedly different from that observed at the cell surface of T2. Regulation of cell surface expression of empty class I molecules thus appears to be regulated not only in the ER, but also at or beyond the Golgi level. Reduction of culture temperature did not result in accumulation of class I material in the *cis*-Golgi area (data not shown), as has been reported for another cell line (17), a finding interpreted to be due to recycling of empty class I molecules between ER and the Golgi complex. The exact site at which class I molecules combine with peptide could therefore include the Golgi complex, and not just the ER, as is commonly assumed.

Our data would be compatible with a model in which the quality control of class I molecules in the biosynthetic pathway utilizes peptide occupancy as a criterion. Dissociation of chaperonin-type proteins may also serve this function. In any case, the disturbance of the ratio of HLA-A locus to HLA-B locus products is not detectable in the ER and Golgi complex of T2, while readily observed at the cell surface. We therefore propose that this quality control is not limited to the ER, but extends to the Golgi complex as well.

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Address correspondence to H. L. Ploegh, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, which is also H.-M. van Santen's present address. P. J. Peters' present address is the Cell Biology and Metabolism Branch, National Institutes of Health, Bethesda, MD 20892.

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