Peptide Influences the Folding and Intracellular Transport of Free Major Histocompatibility Complex Class I Heavy Chains

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Summary

Class I major histocompatibility complex molecules require both β_2-microglobulin (β_2m) and peptide for efficient intracellular transport. With the exception of H-2D^b and L^d, class I heavy chains have not been detectable at the surface of cells lacking β_2m. We show that properly conformed class I heavy chains can be detected in a terminally glycosylated form indicative of cell surface expression in H-2^b, H-2^d, and H-2^s β_2m$^{-/-}$ concanavalin A (Con A)–stimulated splenocytes incubated at reduced temperature. Furthermore, we demonstrate the presence of K^b molecules at the surface of β_2m$^{-/-}$ cells cultured at 37°C. The mode of assembly of class I molecules encompasses two major pathways: binding of peptide to preformed "empty" heterodimers, and binding of peptide to free heavy chains, followed by recruitment of β_2m. In support of the existence of the latter pathway, we provide evidence for a role of peptide in intracellular transport of free class I heavy chains, through analysis of Con A–stimulated splenocytes from transporter associated with antigen processing 1 (TAP1)$^{-/-}$, β_2m$^{-/-}$, and double-mutant TAP1/β_2m$^{-/-}$ mice.

MHC class I molecules present short peptide fragments derived from proteins synthesized in the cytosol to specific CTL (1). The MHC class I molecule is a heterotrimer comprised of a polymorphic transmembrane glycoprotein (heavy chain), β_2-microglobulin (β_2m),[1] and peptide (2). Assembly of the MHC class I molecule takes place in the lumen of the endoplasmic reticulum (ER) and requires the presence of the transporter associated with antigen processing (TAP) 1/TAP2 heterodimer, a protein complex that transports peptides produced in the cytosol into the ER (3, 4). In the absence of a functional TAP complex, heavy chain–β_2m heterodimers are formed that are largely retained in the ER, although their cell surface expression can be partially rescued by addition of peptide to the culture medium (5) or incubation at low temperature (6).

In β_2m-deficient cells, the vast majority of class I heavy chains are also retained in the ER and are rapidly degraded (7). However, limited cell surface expression of H-2D^b molecules has been observed in lymphoid cells from mice lacking β_2m (8). Cell surface–disposed non–β_2m-associated ("free") D^b heavy chains were shown to exhibit conformation-sensitive epitopes and sensitize target cells for killing by alloreactive CTL (9). Nevertheless, in studies with the β_2m-deficient cell line R1E transfected with D^b or K^b, only D^b molecules were detectable at the cell surface (7). The L^d molecule, which is quite similar to D^b (94% identity), has also been shown to be expressed at the surface of β_2m-deficient cells (10).

Are other class I molecules present at the surface of β_2m$^{-/-}$ cells, but at levels below the threshold of available biochemical methods? H-2^b cells deficient for β_2m stimulate K^b-allospecific and -restricted CD8$^+$ T cells (11, 12). Fetal thymic organ cultures (FTOC) prepared from β_2m$^{-/-}$ mice support positive selection of CD8$^+$ T cells expressing a K^b- restricted TCR in the presence of added human β_2m and peptide (13), implying that free K^b heavy chains are present at the cell surface. By pulse–chase analysis of Con A–stimulated splenocytes from H-2^b, H-2^d, and H-2^s mice bred onto a β_2m$^{-/-}$ background, we show here that intracellular transport of class I heavy chains in β_2m$^{-/-}$ cells can be demonstrated for most class I alleles. Specifically, properly conformed K^b heavy chains can be detected on the surface of H-2^b β_2m$^{-/-}$ cells.

Do class I heavy chains assemble with peptide in cells lacking β_2m? Formally, the assembly of MHC class I molecules may proceed either by the binding of peptide to an "empty" heavy chain–β_2m intermediate, or by association of β_2m with

[1] *Abbreviations used in this paper:* αH-2, rabbit αH-2^b antiserum; αHC, anti-heavy chain serum; αp8, K^b-specific antipeptide serum; β_2m, β_2-microglobulin; 1D, one-dimensional; ER, endoplasmic reticulum; FTOC, fetal thymic organ cultures; Staph. A, 10% fixed *Staphylococcus aureus*; TAP, transporter associated with antigen processing.

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heavy chain–peptide complexes. Studies on the human cell lines T1 and the TAP-deficient T2 have shown that the HLA-B5 heavy chains readily assemble with β_2m in the absence of TAP-dependent peptides (14), and have suggested that, even in the presence of these peptides, empty complexes may form before peptide binding (14, 15). Although these data suggest that association of the heavy chain with β_2m usually precedes peptide binding, free D^b heavy chains in detergent lysate of $\beta_2m^{-/-}$ cells can be induced to fold upon addition of peptide alone (16). Thus, in the course of biosynthesis, a portion of class I molecules might assemble by forming a complex with peptide first, followed by recruitment of β_2m. By comparing the conformation and cell surface expression of heavy chains in Con A–stimulated splenocytes from H-2^b $\beta_2m^{-/-}$ and H-2^b TAP1/$\beta_2m^{-/-}$ mice, we demonstrate that the folding and intracellular transport of free class I heavy chains is enhanced by TAP-dependent peptides.

Materials and Methods

Antibodies. The following antisera and mAbs, prepared as tissue culture supernatants, were used: rabbit αH-2^b (αH-2; recognizes conformed H-2K, D, and L locus products; a gift of Dr. S. Nathenson, Albert Einstein College of Medicine, New York), B22.249 (α1 of D^b; 17), 28-14-8s (α3 of D^b; 18), Y3 (α1/α2 of K^b; 19), K10.56.1 (specific for conformed K^b; a gift of Dr. G. Waneck, Massachusetts General Hospital Cancer Center, Charlestown, MA), rabbit anti-p8 (recognizes the cytoplasmic tail of K^b; prepared in our laboratory essentially as described; 20), and rabbit αHC, a rabbit polyclonal antiserum directed against free class I heavy chains. The latter was generated by immunizing New Zealand white rabbits with inclusion bodies (kindly provided by Dr. S. Nathenson) of soluble K^b or D^b heavy chains produced in bacteria by overexpression. Before immunoprecipitations, antibodies were prebound to 50 μL 10% fixed *Staphylococcus aureus* (Staph.A) for 60 min and then washed three times in ice-cold wash buffer (0.5% NP-40, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) to remove excess antibody and serum proteins. For FACS® stainings, antibodies were purified via protein A-Sepharose (Repligen Corp., Cambridge, MA).

Mice. The generation of TAP1$^{-/-}$ and $\beta_2m^{-/-}$ (H-2^b haplotype) mice has been described (21, 22). $\beta_2m^{-/-}$ mice of H-2^b haplotype (C57BL/6X129/Ola) were a gift from Dr. B. Koller (University of North Carolina, Durham, NC). $\beta_2m^{-/-}$ mice of H-2^d haplotype were prepared by crossing H-2^b $\beta_2m^{-/-}$ mice with BALB/c mice (H-2^d), followed by a cross with B10.D2 mice and five subsequent generations of brother-sister matings (mice were a gift from Dr. T. Hansen, Washington University School of Medicine, St. Louis, MO). H-2^s $\beta_2m^{-/-}$ mice were generated by crossing with SJL/J (H-2^s) mice, followed by 11 backcrosses (mice were a gift from Dr. D. Roopenian, The Jackson Laboratory, Bar Harbor, ME). To generate TAP1/β_2m double-mutant mice (designated TAP1/$\beta_2m^{-/-}$), TAP1$^{-/-}$ and $\beta_2m^{-/-}$ mice (both H-2^b) were mated, and offspring were subsequently intercrossed and typed. Control mice heterozygous for both the TAP1 and β_2m genes were generated from the same crosses or by mating TAP1/$\beta_2m^{-/-}$ mice with B6 mice. All other control mice used were obtained from The Jackson Laboratory. All mice used were at the age of 6–12 wk. Mice were maintained at the Division of Comparative Medicine, Center for Cancer Research, Massachusetts Institute of Technology (Cambridge, MA), in accordance with institutional guidelines.

Gel Electrophoresis. One-dimensional (1D) IEF was performed as described (23, 24). Fluorography was accomplished by impregnating the gels with 2,5-diphenyloxazol in DMSO before exposure to film (XAR-5; Eastman Kodak Co., Rochester, NY).

Immunoprecipitations. 10^8 splenocytes were stimulated with Con A (2.5 μg/ml) in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS, L-glutamine (2 mM), penicillin (1:1,000 dilution U/ml), and streptomycin (100 μg/ml) for 48 h before labeling. After a 45-min starvation in methionine/cysteine-free medium, the cells were pulsed with 500 μCi/ml [^{35}S]methionine/cysteine (80/20) for 15 min, and then chased in complete medium containing 1 mM nonradioactive methionine and cysteine, at either 37°C or 26°C. Aliquots of cells (10^7) were spun down for each chase point and lysed in 0.5 ml ice-cold lysis buffer (0.5% NP-40, 50 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM PMSF). After 45 min on ice, the lysates were centrifuged to remove the nuclei and cell debris, and then precleared twice for 60 min (4°C) with 2 μl normal rabbit serum prebound to 100 μl Staph. A suspension before specific immunoprecipitation. To minimize antibody carryover between sequential immunoprecipitations, lysates were incubated for 30 min with 50 μl Staph. A between immunoprecipitation steps. The Staph. A pellets were washed three times in ice-cold wash buffer before analysis by 1D-IEF.

Cell Surface Iodinations. Con A–stimulated splenocytes from normal and H-2^b $\beta_2m^{-/-}$ mice were prepared as described above. Before iodination, dead cells were removed by centrifugation with Lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Canada) according to the manufacturers protocol. Cells (10^7) were surface labeled with 1.0 mCi of $Na^{125}I$ (1 Ci = 37 GBq) in ice-cold PBS by lactoperoxidase-catalyzed iodination. Class I molecules were subsequently immunoprecipitated as described above.

Flow Cytometry. Splenocytes were stimulated in serum-free medium (AIM-V; GIBCO BRL) supplemented with 250 ng/ml Con A for 36 h at 37°C, followed by incubation at either 37° or 26°C for an additional 16 h. Cells incubated at 26°C were buffered by the addition of 20 mM Hepes, pH 7.4. For cell surface staining, 10^6 cells were aliquoted per antibody. Cells were incubated on ice for 30 min with 10–30 μg/ml of purified Y3 mAb (αK^b), B22.249 mAb (αD^b), or rabbit αHC serum (αK^b, D^b), washed once with cold PBS, and then incubated for 30 min with a 1:400 dilution of goat anti–mouse or anti–rabbit FITC-coupled antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). Quantitation of cell staining was performed on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA), with propidium iodide used to gate out dead cells. Background staining values were obtained by incubating cells with 10 μg/ml purified IgG_{2a} (for B22.249), IgG_{2b} (for Y3), or preimmune serum (for rabbit αHC) before incubation with the FITC-conjugated secondary Ab.

Results

Folding and Intracellular Transport of Class I Heavy Chains in H-2^b H-2^d, and H-2^s $\beta_2m^{-/-}$ Cells. To generate an antiserum against class I MHC heavy chains that would recognize epitopes normally obscured by β_2m or present only on unfolded heavy chains, we immunized rabbits with either K^b or D^b inclusion bodies produced by expression in bacteria. Initial characterization of the anti-K^b and -D^b sera showed that both reacted equally well against K^b and D^b heavy chains in immunoprecipitations, immunoblots, and ELISA (data not shown). The antisera were combined for the experiments described below, and this preparation is re-

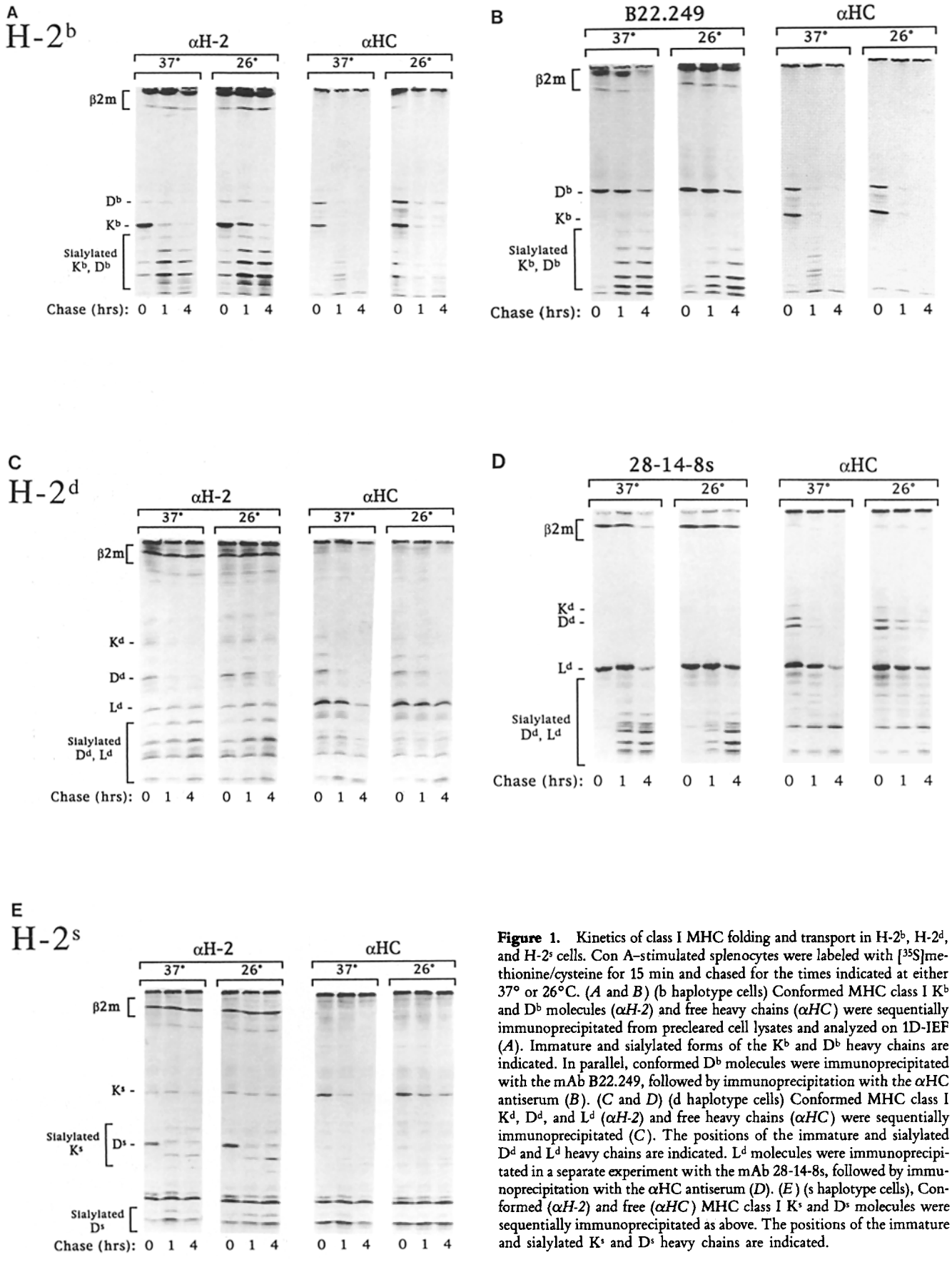

Figure 1. Kinetics of class I MHC folding and transport in H-2^b, H-2^d, and H-2^s cells. Con A–stimulated splenocytes were labeled with [^{35}S]methionine/cysteine for 15 min and chased for the times indicated at either 37° or 26°C. (*A* and *B*) (b haplotype cells) Conformed MHC class I K^b and D^b molecules (*αH-2*) and free heavy chains (*αHC*) were sequentially immunoprecipitated from precleared cell lysates and analyzed on 1D-IEF (*A*). Immature and sialylated forms of the K^b and D^b heavy chains are indicated. In parallel, conformed D^b molecules were immunoprecipitated with the mAb B22.249, followed by immunoprecipitation with the αHC antiserum (*B*). (*C* and *D*) (d haplotype cells) Conformed MHC class I K^d, D^d, and L^d (*αH-2*) and free heavy chains (*αHC*) were sequentially immunoprecipitated (*C*). The positions of the immature and sialylated D^d and L^d heavy chains are indicated. L^d molecules were immunoprecipitated in a separate experiment with the mAb 28-14-8s, followed by immunoprecipitation with the αHC antiserum (*D*). (*E*) (s haplotype cells), Conformed (*αH-2*) and free (*αHC*) MHC class I K^s and D^s molecules were sequentially immunoprecipitated as above. The positions of the immature and sialylated K^s and D^s heavy chains are indicated.

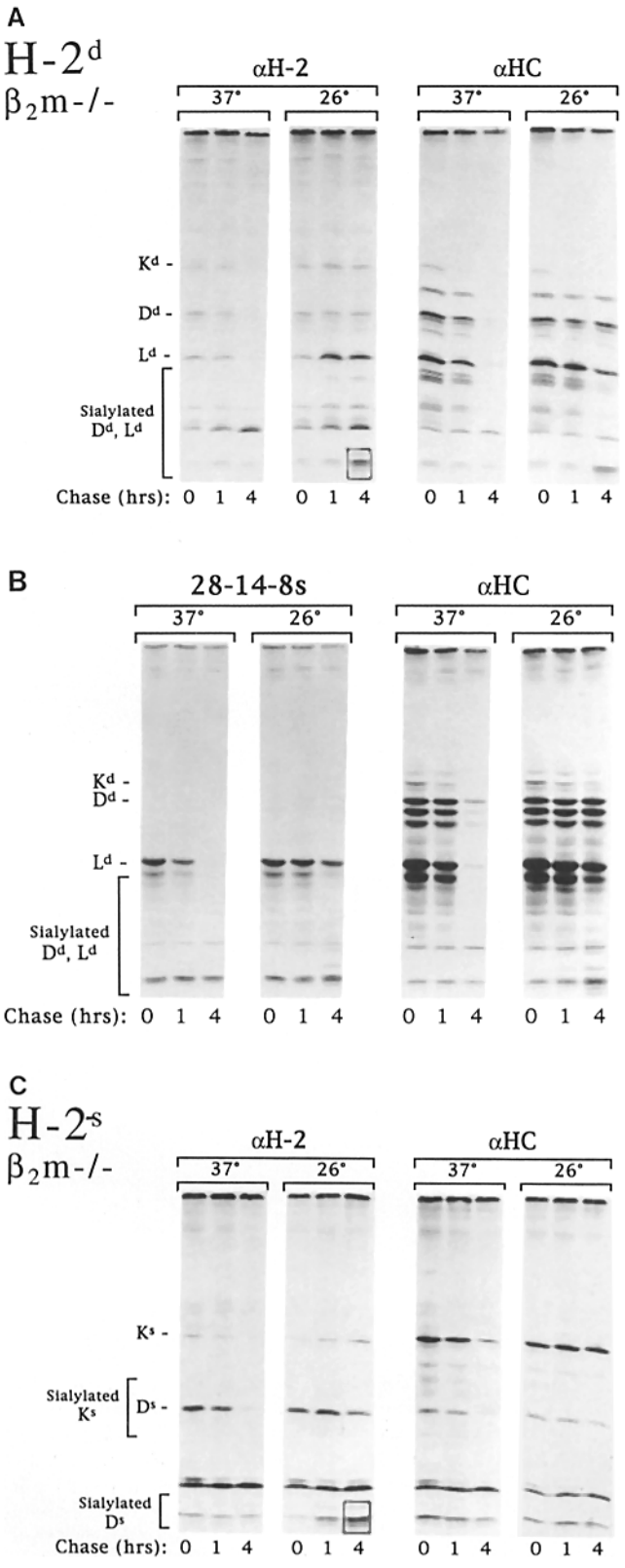

Figure 2. Kinetics of class I MHC transport in H-2^d and H-2^s $\beta_2m^{-/-}$ cells. Con A–stimulated splenocytes were pulse labeled as described above and chased for the times indicated at 26° or 37°C. (*A* and *B*) Conformed class I K^d, D^d, and L^d (*αH-2*), and unfolded heavy chains (*αHC*) were sequentially immunoprecipitated and resolved on 1D-IEF (*A*). Sialylated forms of L^d and D^d that emerge at 26°C are boxed. In a separate experiment, L^d molecules alone (*28-14-8s*) were first immunoprecipitated, followed by αHC immunoprecipitations (*B*). The positions of the immature and sialylated K^d, D^d, and L^d molecules are indicated. (*C*) Conformed (*αH-2*) and unfolded (*αHC*) class I K^s and D^s molecules were sequentially immunoprecipitated and analyzed on 1D-IEF. Immature and sialylated K^s and D^s molecules (*box*) are indicated.

ferred to as αHC. The αHC serum showed reactivity against class I heavy chains from H-2K, D, and L locus products of all haplotypes tested (b, d, s, k, f), and also reacted with human class I heavy chains in immunoblots, as do anti–free class I heavy chain reagents produced against HLA-A, B, and C heavy chains (25). This characterization revealed that the αHC serum reacts broadly with non–β_2m-associated ("free") class I heavy chains irrespective of conformation, peptide content, or glycan modification; no reactivity with heavy chain–β_2m complexes was observed in immunoprecipitations on K^b and D^b molecules from biosynthetically labeled cell lines (data not shown), including RMA and its TAP-2 deficient counterpart RMA-S (26). Trace amounts of coprecipitating β_2m were occasionally observed in immunoprecipitates from H-2^d cells (see Fig. 1 *D*).

To follow the fate of properly conformed and unfolded class I heavy chains in the presence or absence of β_2m, we performed a series of pulse–chase experiments on Con A–stimulated splenocytes prepared from H-2^b, H-2^d, and H-2^s mice (Fig. 1) and their $\beta_2m^{-/-}$ counterparts (Fig. 2 and see Fig. 4, *C* and *D*). We included in our analysis cells chased at 26°C to determine the effect of low temperature on the stability and conformation of free heavy chains (6). Aliquots of cells were pulsed labeled for 15 min, chased at either 37° or 26°C, and immunoprecipitated first with a conformation-sensitive antibody (e.g., αH-2 serum), followed by a second round of immunoprecipitation with the αHC serum. We analyzed the immunoprecipitates by 1D-IEF to resolve the complex mixture of biosynthetic intermediates. In the course of maturation, class I molecules acquire negatively charged sialic acid residues, which results in a shift in isoelectric point toward the anode. Sialylation is a marker for transport through the *trans*-Golgi network to the cell surface, and those heavy chains that are not sialylated reside in the ER or proximal to the *trans*-Golgi network (27). The nonsialylated class I heavy chains are thus referred to as "immature."

Fig. 1 *A* shows a pulse chase of H-2^b Con A–stimulated splenocytes analyzed with the αH-2 and αHC antisera. The bulk of the assembled K^b and D^b is rapidly sialylated at 26° and 37°C, although more of the immature complexes are detectable during the 26°C chase (compare 1-h time points). The subsequent immunoprecipitation with αHC reveals a transient pool of immature free K^b and D^b at the onset of the chase, most of which disappears by 1 h of chase. Some sialylated free heavy chains appear after 1 h at 37°C, most likely from dissociation of unstable complexes (see below), but these are no longer detectable by 4 h. Because the αH-2

antiserum recognizes both conformed K^b and D^b (the latter less efficiently), we performed a parallel immunoprecipitation with the mAb B22.249, which recognizes a conformation-dependent epitope on the $\alpha 1$ domain of D^b (17). Consistent with earlier reports (28), D^b molecules were transported with slower kinetics than K^b (compare 0-min time points, 37°C, Fig. 1, *A* and *B*), and less completely (4-h time points, 37°C, αH-2 and B22.249 panels).

We performed a similar analysis on Con A–stimulated splenocytes from H-2^d and H-2^s haplotype mice (Fig. 1, *C–E*) to serve as a comparison for experiments performed on $\beta_2m^{-/-}$ mice of these haplotypes (described below). Both K^d and D^d heavy chains were efficiently assembled and transported after 1 h of chase at 37°C, with somewhat slower kinetics at 26°C (Fig. 1 *C*). In contrast, free L^d heavy chains, as well as immature, conformed L^d molecules, were detectable throughout the chase, even at 37°C (Fig. 1 *C*, αHC and αH-2 4-h time points). To follow only L^d molecules, we repeated the pulse–chase experiment and immunoprecipitated first with the L^d-specific mAb 28-14-8s, followed by immunoprecipitation with αHC serum (Fig. 1 *D*). The rate of transport of L^d is similar to that of the closely related D^b molecule (compare Fig. 1, *B* and *D*, B22.249 and 28-14-8s immunoprecipitates), but in contrast to D^b, immature free L^d heavy chains persist throughout the chase, even at 37°C (Fig. 1 *D*). Trace amounts of β_2m can be seen in the αHC immunoprecipitates (Fig. 1 *D*), suggesting that αHC may also recognize a minor population of class I heavy chains that are loosely associated with β_2m (e.g., L^dalt; 10). Direct αHC immunoprecipitation of class I heavy chains from lysates of metabolically labeled Con A blasts (H-2^d haplotype) also coprecipitated minute amounts of β_2m (data not shown).

Analysis of H-2^s cells indicates that both K^s and D^s are transported less completely than their H-2^b counterparts (Fig. 1 *E*, 4-h time points). Very little immature free D^s is detectable at either chase temperature, even at 0 min, although small amounts of sialylated free D^s heavy chains are evident at 1 h (37°C chase). The apparent absence of free D^s heavy chains is not due to lack of reactivity with the αHC antiserum, as demonstrated by pulse–chase analysis of H-2^s $\beta_2m^{-/-}$ cells (Fig. 2; see below). We conclude that rates of class I assembly and intracellular transport show characteristic differences among the alleles examined (29).

With the exception of D^b (7) and L^d (10), transport of class I heavy chains in the absence of β_2m has not been observed. We performed pulse–chase experiments as described above on Con A–stimulated splenocytes from H-2^b, H-2^d, and H-2^s mice devoid of β_2m and analyzed the immunoprecipitated products by 1D-IEF to score for sialylation of class I heavy chains. Unexpectedly, the αH-2 antiserum recognized small amounts of class I material in all three $\beta_2m^{-/-}$ haplotypes (Fig. 2, *A* and *C*, and see Fig. 4, *C*), implying that conformed free heavy chains are present in the absence of β_2m. Does the polyclonal αH-2 antiserum contain some reactivity against unfolded free heavy chains, which could account for our results? This does not appear to be the case, because reimmunoprecipitation of SDS-denatured K^b molecules with the αH-2 serum recovered only trace amounts of material (data not shown). Similarly, the αH-2 serum does not react with free K^b heavy chains produced by in vitro translation, even in the presence of oxidized glutathione (data not shown; [30]).

No sialylation of either conformed or unfolded H-2^b class

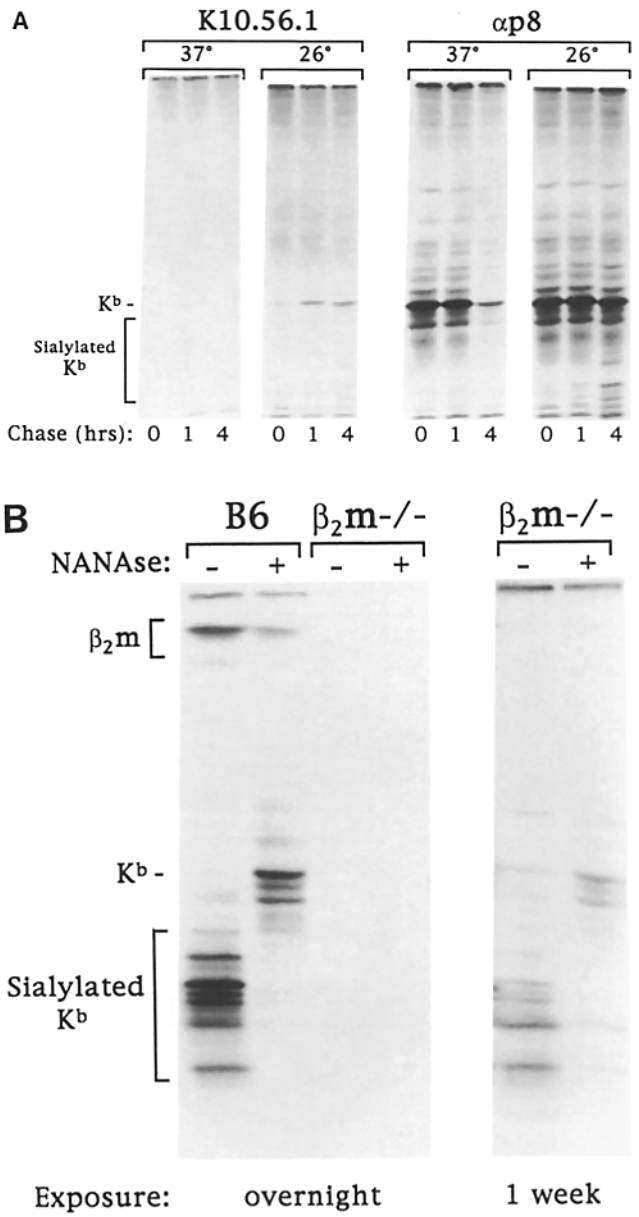

Figure 3. Folding and cell surface expression of K^b molecules in H-2^b $\beta_2m^{-/-}$ cells. (*A*) Con A–stimulated splenocytes from H-2^b $\beta_2m^{-/-}$ mice were pulse labeled as described above and chased for the times indicated at 26° or 37°C. Conformed K^b molecules were immunoprecipitated with the mAb K10.56.1, followed by immunoprecipitation of total K^b with αp8, and resolved on 1D-IEF. (*B*) Con A–stimulated splenocytes prepared from B6 and H-2^b $\beta_2m^{-/-}$ mice were surface iodinated, following by immunoprecipitations of total K^b with αp8. The immunoprecipitates were either digested with neuraminidase (+) or kept on ice (−) before 1D-IEF analysis. Labeled K^b molecules from H-2^b $\beta_2m^{-/-}$ cells are apparent in the week-long exposure.

I heavy chains in H-2^b β_2m$^{-/-}$ cells was observed after 4 h of chase at 37°C (7); remarkably, transport of free heavy chains was partially rescued by lowering the chase temperature to 26°C (see Fig. 4, *C* and *D*, 4-h time points). The sialylated heavy chains immunoprecipitated with the αH-2 serum after 4 h of chase include both K^b and D^b, as shown by a comparison with the parallel B22.249 (D^b) immunoprecipitation (see Fig. 4, *D*, 4- h time points, 26°C). Sialylated K^b molecules are also detectable in K10.56.1 (recognizes conformed K^b; Fig. 3 *A*), p8 (recognizes all K^b; Fig. 3 *A*), and Y3 (recognizes conformed K^b; data not shown) immunoprecipitates of β_2m$^{-/-}$ cells chased at 26°C. Thus, by analogy to earlier work on the TAP2-deficient cell line RMA-S, we find that low temperature favors the cell surface accumulation of K^b heavy chains in the absence of β_2m.

In light of the dramatic effect of temperature on the degradation of free class I heavy chains in β_2m$^{-/-}$ cells (Fig. 4 *C*, αHC, compare 37° and 26°C 4-h time points), we considered it likely that the heavy chains are transported to the cell surface at 37°C, but are rapidly degraded such that the steady levels are too low to be detected by metabolic labeling. To assay more directly for cell surface–disposed K^b molecules at 37°C, we performed iodinations of H-2^b wild-type and β_2m$^{-/-}$ cells and immunoprecipitated K^b heavy chains with the anti-p8 serum (Fig. 3 *B*). Analysis by 1D-IEF showed that K^b heavy chains are present at the surface of β_2m$^{-/-}$ cells, albeit at levels well below that observed in nonmutant H-2^b cells, as expected (Fig. 3, *B*, compare overnight with 1-wk exposure). Digestion of the immunoprecipitated K^b material with neuraminidase confirmed that the bands observed on 1D-IEF correspond to sialylated K^b molecules (Fig. 3 *B*, − and + lanes). We conclude that low levels of free K^b heavy chains are expressed on the cell surface in the absence of β_2m at 37°C.

Are free class I heavy chains transported to the cell surface in other H-2 haplotypes? In H-2^d β_2m$^{-/-}$ cells chased at 26°C (Fig. 2, *A* and *B*), degradation of heavy chains is reduced, and sialylated L^d and D^d are apparent by the end of the chase (Fig. 2 *A*, 4-h time points). The induction of sialylated L^d at 26°C is visualized more clearly with the mAb 28-14-8s (Fig. 2 *B*, 4-h time points). In H-2^s β_2m$^{-/-}$ cells, K^s molecules remain largely as nonconformed immature heavy chains at both chase temperatures, whereas D^s, like L^d, is partially sialylated after 4 h of chase at 26°C (Fig. 2 *C*). Thus, by incubating cells at 26°C, we can detect intracellular transport of free class I heavy chains other than D^b and L^d in H-2^b, H-2^d, and H-2^s β_2m$^{-/-}$ cells. In particular, we observe transport of K^b molecules in the absence of β_2m, which provides a biochemical basis for the K^b-restricted T cell selection observed in β_2m$^{-/-}$ FTOC (13).

Peptide Affects the Folding and Intracellular Transport of Free Class I Heavy Chains. To assess the relative contributions of the TAP1/TAP2 complex and β_2m to the folding and intracellular transport of class I heavy chains, we performed pulse–chase experiments as described above on H-2^b Con A–stimulated splenocytes lacking either TAP1 (TAP1$^{-/-}$), β_2m (β_2m$^{-/-}$), or both (TAP1/β_2m$^{-/-}$). Immunoprecipitations of K^b and D^b class I molecules were performed with either αH-2 or B22.249, followed by immunoprecipitation with the αHC serum, and then analyzed on 1D-IEF (Fig. 4). In cells lacking the TAP1 gene, class I transport was impaired at 37°C, but partially rescued at 26°C (Fig. 4 *A*), consistent with previous reports (21). In contrast to normal H-2^b cells (Fig. 1), free heavy chains in the TAP1$^{-/-}$ background persist throughout the chase and are present in amounts approximately equal to that of β_2m-associated material (Fig. 4 *A*). Sialylated free heavy chains, which appear only during the 37°C chase, most likely arise from dissociation of thermolabile complexes in the course of intracellular transport. We failed to detect sialylation of B22.249-immunoprecipitable D^b molecules at either temperature in TAP1$^{-/-}$ cells (Fig. 4 *B*).

Cells lacking β_2m are more deficient in intracellular transport (see above) and folding of class I heavy chains than TAP1-deficient cells (compare Fig. 4, *A* and *C*). Like H-2^d and H-2^s class I heavy chains (Fig. 2), K^b and D^b are present predominantly as nonconformed free heavy chains in the β_2m$^{-/-}$ background and are degraded by 4 h at 37°C (Fig. 4 *C*). The breakdown of free heavy chains is strongly inhibited at 26°C (αHC, 4-h time points), and a greater proportion of both K^b and D^b appear to fold into a native-like conformation (Fig. 4 *C*, αH-2, and *D*, B22.249, 1-h time points). Little, if any, folded K^b is detectable by the conformation-sensitive mAb K10.56.1 during the chase at 37°C, but at 26°C, conformed free K^b heavy chains are detectable by 1 h (Fig. 3, 26° time points), in agreement with the results obtained with the αH-2 serum. The appearance of folded K^b molecules at 26°C is not a consequence of impaired heavy chain degradation, since the total amount of K^b material (*αp8*) after 1 h of chase at 37° or 26°C is identical (Fig. 3 *A*). We conclude that, at low temperature, free K^b heavy chains can acquire a conformation similar to that observed for properly assembled class I molecules.

Assembly of class I molecules in normal cells may proceed via peptide binding to "empty" heavy chain–β_2m heterodimers, or alternatively, by association of β_2m with heavy chain–peptide complexes (31). To address the question of whether TAP-dependent peptides affect the folding and cell surface expression of free class I heavy chains, we crossed H-2^b TAP1$^{-/-}$ and β_2m$^{-/-}$ mice to obtain double-mutant mice (TAP1/β_2m$^{-/-}$). In Con A– stimulated splenocytes lacking both β_2m and TAP1, the distribution of class I heavy chains between free and conformed populations is similar to that of β_2m$^{-/-}$ cells, with the bulk of the K^b or D^b heavy chains in an unfolded conformation (αHC reactive). Loss of TAP1 affects the folding of D^b more profoundly than K^b molecules, judging from the ratios of D^b and K^b immunoprecipitated with the mAb B22.249 and αH-2 antiserum, respectively, in the two cell types (compare Fig. 4, *D* and *F*, for D^b and Fig. 4, *C* and *E* for K^b, 1-h time points). The most striking difference between the β_2m$^{-/-}$ and double-mutant cells is that no sialylation of either K^b or D^b is observed in the latter, even at 26°C (Fig. 4, *E* and *F*). Extending the chase of 8 h at 26°C still did not result in any detectable

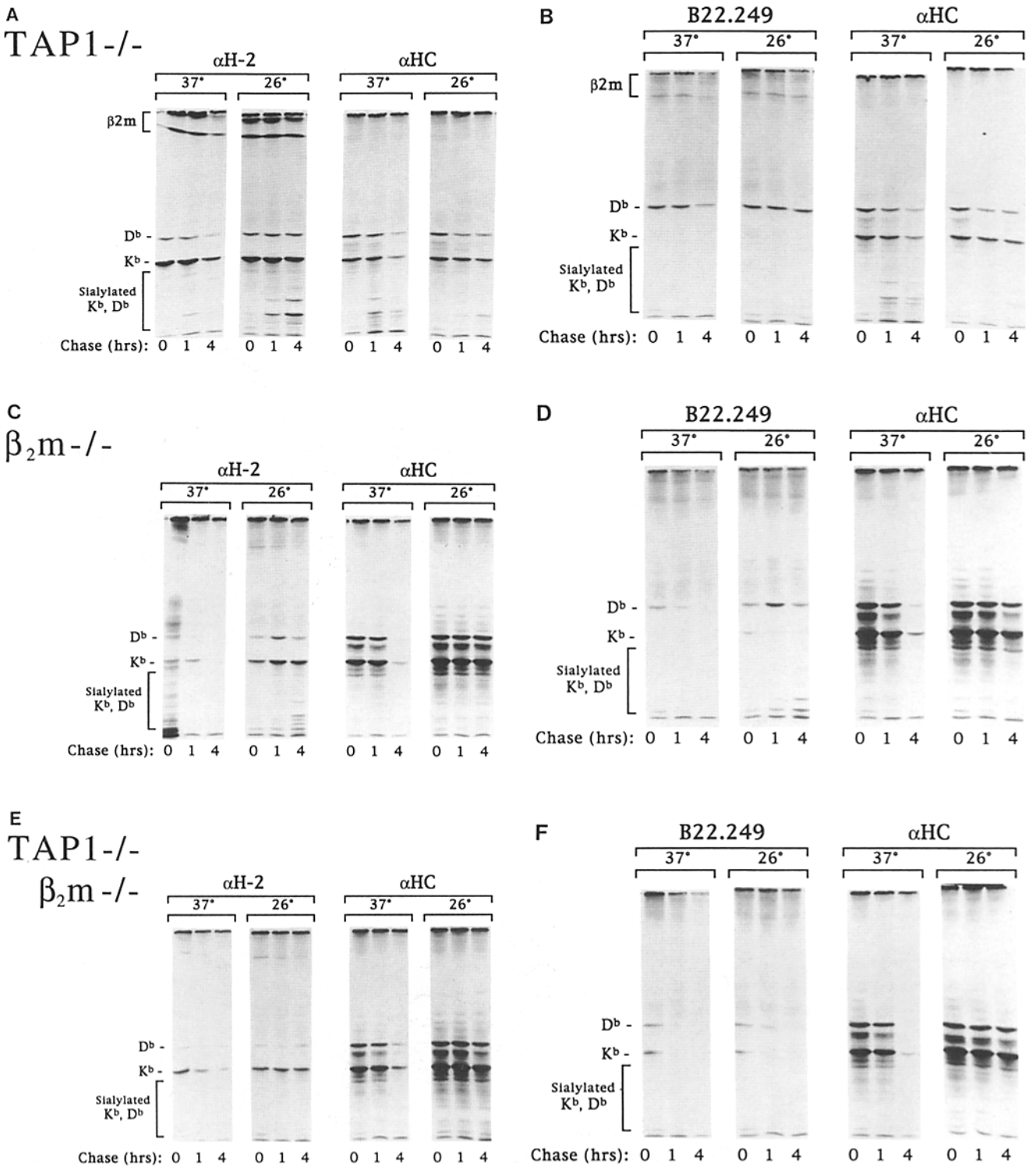

Figure 4. Kinetics of class I MHC transport in H-2^b TAP1$^{-/-}$, β_2m$^{-/-}$, and TAP1/β_2m$^{-/-}$ cells. Con A–stimulated splenocytes were pulse labeled as described above and chased for the times indicated at 37° or 26°C. Conformed class I K^b and D^b (*αH-1*; *A*, *C*, and *E*), and D^b heavy chains alone (*B22.249*; *B*, *D*, and *F*), were immunoprecipitated in parallel, followed by αHC immunoprecipitations. Immature and sialylated class I molecules were resolved in 1D-IEF. (*A* and *B*) TAP1$^{-/-}$ cells. (*C* and *D*) β_2m$^{-/-}$ cells. The 0-min αH-2 time point (37°C) contains atypical background due to an aspirator malfunction. (*E* and *F*) TAP1/β_2m$^{-/-}$ cells. Note the appearance of sialylated K^b and D^b in β_2m$^{-/-}$, but not in TAP1/β_2m$^{-/-}$ cells, after 4 h of chase at 26°C.

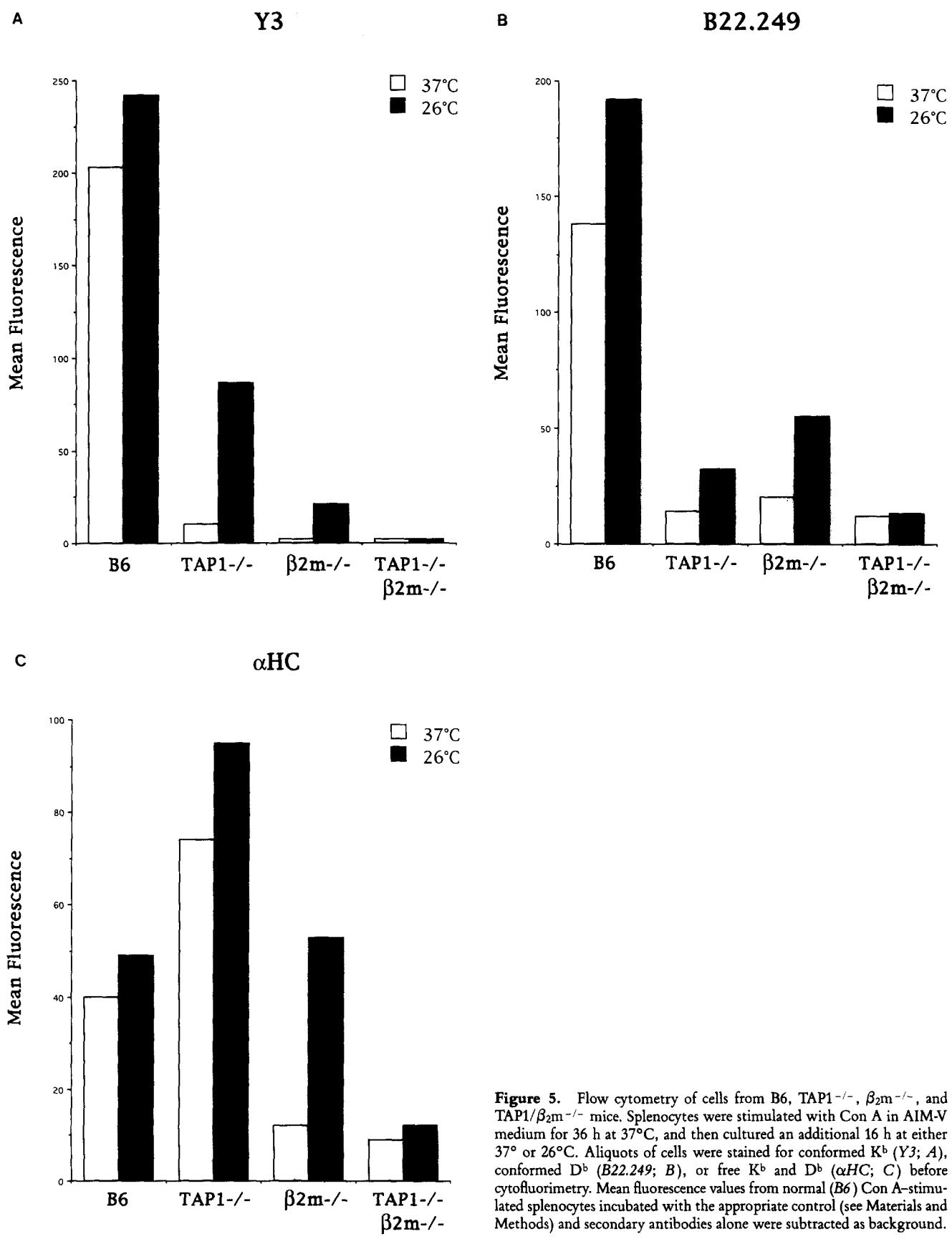

Figure 5. Flow cytometry of cells from B6, TAP1$^{-/-}$, β_2m$^{-/-}$, and TAP1/β_2m$^{-/-}$ mice. Splenocytes were stimulated with Con A in AIM-V medium for 36 h at 37°C, and then cultured an additional 16 h at either 37° or 26°C. Aliquots of cells were stained for conformed K^b (*Y3*; *A*), conformed D^b (*B22.249*; *B*), or free K^b and D^b (*αHC*; *C*) before cytofluorimetry. Mean fluorescence values from normal (*B6*) Con A–stimulated splenocytes incubated with the appropriate control (see Materials and Methods) and secondary antibodies alone were subtracted as background.

sialylation of K^b or D^b (data not shown). Within the limits of detection of this experiment, we conclude that the presence of a functional TAP1/TAP2 complex is required for intracellular transport of free class I heavy chains.

Cytofluorimetry of Class I Heavy Chains at the Surface of Normal and Mutant Lymphoid Cells. As an adjunct to our pulse–chase analyses, we measured class I surface expression at steady state by flow cytometric analysis of Con A–stimulated splenocytes from normal (B6), TAP1$^{-/-}$, $\beta_2m^{-/-}$, and TAP1/$\beta_2m^{-/-}$ mutant mice (all H-2^b haplotype) with the Y3 (αK^b) and B22.249 (αD^b) mAbs, and the gamma globulin fraction from the αHC serum (αK^b, D^b). Fig. 5 *A* shows the relative proportion of Y3-reactive cell surface class I heavy chains in the four cell types. At 37°C, TAP1$^{-/-}$ cells express conformed K^b molecules at 5% of the levels observed in normal cells, whereas at 26°C, the relative expression increases to 35%, consistent with previous reports (6). Neither $\beta_2m^{-/-}$ nor TAP1/$\beta_2m^{-/-}$ cells express detectable amounts of conformed K^b molecules at 37°C; however, $\beta_2m^{-/-}$ cells cultured at 26°C show a modest rescue of K^b expression to 10% of wild-type levels. The absence of any detectable K^b expression in the double-knockout cells, even at 26°C, is consistent with the pulse–chase experiments described earlier (Fig. 4). While quantitative data have not been presented here, we have observed that the rates of synthesis of class I heavy chains, as assessed in biosynthetic labeling experiments, are indistinguishable for the four cell types.

The cell surface expression of conformed D^b molecules in the four cell types was assayed with the mAb B22.249 (Fig. 5 *B*). In contrast to K^b, the expression of properly folded D^b molecules in TAP1$^{-/-}$ cells is only marginally rescued at 26°C (9-fold vs. 2-fold increase in staining), consistent with the pulse–chase experiment shown in Fig. 4 *B*. Interestingly, after incubation at 26°C, $\beta_2m^{-/-}$ cells exhibit greater D^b staining than TAP1$^{-/-}$ cells (1.7-fold), suggesting that the D^b molecule relies more on TAP-dependent peptides than on β_2m to be transported to the cell surface. Cells lacking both β_2m and TAP1 show no increase in staining after incubation at reduced temperature.

Free class I heavy chains (K^b and D^b) were detected on the cell surface by cytofluorimetry with the αHC serum (Fig. 5 *C*). While the loss of TAP1 results in a drastic decrease in expression of conformed class I molecules at 37°C (Fig. 5, *A* and *B*), cells lacking TAP1 express twice the levels of free heavy chains observed in normal cells. Culturing normal or TAP1-deficient cells in the presence of 10% FCS results in a two- to threefold decrease in free heavy chain staining (data not shown), supporting earlier findings that exogenous bovine β_2m can associate with free class I heavy chains at the cell surface (32, 33). In $\beta_2m^{-/-}$ cells, total heavy chain expression is enhanced fivefold by overnight incubation at 26°C (Fig. 5 *C*). Similar to the results obtained with the mAb B22.249, low levels of free heavy chain staining are observed in the double-knockout cells at both temperatures, suggesting that D^b may be expressed at the cell surface, albeit weakly, in the absence of both peptide and β_2m.

Discussion

We have demonstrated the presence of properly conformed H2- K, -D, and -L locus products at the surface of cells lacking β_2m, a finding that generalizes previous observations on D^b and L^d to other class I alleles. Furthermore, we have shown here that the intracellular transport of free heavy chains in $\beta_2m^{-/-}$ cells is dramatically enhanced by the presence of a functional TAP1 gene.

Although many immunochemical reagents directed against mouse class I molecules have been prepared, few recognize non–β_2m-associated heavy chains exclusively. The K^b-specific antipeptide 8 serum (αp8) has been used in combination with conformation-sensitive reagents to document pools of free K^b heavy chains (34, 35), but this antiserum is not specific for free heavy chains. Lie et al. (36) have extensively characterized a partially folded form of the L^d molecule (L^dalt), which is recognized by the 64-3-7 mAb. L^dalt heavy chains are weakly associated with β_2m and can be induced to fold into a native conformation, as defined by the mAb 30-5-7, upon incubation with peptide (10). Here, we introduce a rabbit αHC that reacts primarily with class I molecules when not associated with β_2m. In this respect, the αHC serum is analogous to the human class I reagents rabbit αHC (37), HC10 (37), HCA2 (25), and LA45 (38), all of which are largely, if not exclusively, specific for free heavy chains. We used the αHC serum to define a population of non-β_2m-associated class I heavy chains, both in detergent extracts and at the cell surface, that are immunochemically distinct from heavy chains in a complex with β_2m.

The conformed state of class I molecules is operationally defined here by the presence of epitopes recognized by immunochemical reagents such as the αH-2 serum and the mAb Y3 (19). How does the balance between conformed and unfolded heavy chains depend on peptide and/or β_2m? To address this question, we compared the pools of conformed and unfolded class I heavy chains in H-2^b Con A–stimulated splenocytes deficient for TAP1, β_2m, or both (Fig. 4). We found that TAP1$^{-/-}$ cells express approximately equal amounts of conformed and unfolded K^b and D^b heavy chains, whereas in cells lacking β_2m or both TAP1 and β_2m, the majority of the class I heavy chains are unfolded. Using a panel of conformation-sensitive mAb and antisera, we detected low levels of properly conformed class I molecules in H-2^b (Figs. 3 and 4), H-2^d, and H-2^s (Fig. 2) $\beta_2m^{-/-}$ cells incubated at 37°C. As expected, conformed free heavy chains were unstable under physiological conditions; however, by lowering the incubation temperature to 26°C, the proportion of folded to unfolded heavy chains in $\beta_2m^{-/-}$ cells was increased (Figs. 3 and 4).

To demonstrate the presence of conformed free heavy chains on the cell surface and to address the requirements for intracellular transport of the latter, we analyzed mutant mice lacking either TAP1, β_2m, or both for cell surface class I molecules. The majority of class I complexes synthesized in TAP1$^{-/-}$ cells are functionally "empty," based on their in-

stability in the absence of exogenous peptides (5) and thermolability (6). As anticipated, we found that free class I heavy chains were expressed on the surface of TAP1$^{-/-}$ cells at high levels relative to normal H-2^b cells (Fig. 5), presumably as a consequence of increased dissociation of class I complexes in the absence of TAP-dependent peptides. In contrast to K^b, we found that the expression of D^b molecules in TAP-deficient cells was only rescued to a minor extent upon incubation at 26°C (Figs. 4 and 5), suggesting that "empty" D^b molecules are less competent for intracellular transport than their K^b counterparts.

Accumulation of free class I heavy chains at the surface of β_2m$^{-/-}$ cells has been observed only for D^b and L^d molecules (7, 9, 10). By cell surface iodination, we observed K^b heavy chains at the surface of β_2m$^{-/-}$ cells incubated at 37°C (Fig. 3). We detected sialylation of class I heavy chains in H-2^b, H-2^d, and H-2^s β_2m$^{-/-}$ cells (Figs. 2 and 4) chased at reduced temperature (26°C). Low temperature partially rescued the folding (Fig. 3), inhibited degradation (Figs. 3 and 4), and increased cell surface levels (Fig. 5) of free K^b and D^b heavy chains. The relationship between folding and intracellular transport of class I molecules is well established (39); thus, it is not surprising that we observed increased intracellular transport of K^b molecules under conditions that favor the folding of the latter. We conclude that conformed free class I heavy chains are expressed on the surface of β_2m$^{-/-}$ cells, thus demonstrating that β_2m is not strictly necessary for the transport of class I molecules.

Free D^b heavy chains in detergent lysates have been shown to fold into a nativelike conformation upon addition of peptide (16). In β_2m$^{-/-}$ cells incubated at low temperature, some proportion of free heavy chains may bind peptide in the ER and thereby escape retention by calnexin (40). We observed that Con A–stimulated splenocytes from the double-mutant (TAP1/β_2m$^{-/-}$) mice did not express detectable levels of either sialylated K^b or D^b, even at reduced temperature (Fig. 4). Likewise, we failed to detect either conformed or unfolded class I heavy chains at the surface of double-mutant cells by surface iodination (data not shown). When analyzed by cytofluorimetry, double-mutant cells exhibited very low levels of staining with D^b-reactive antibodies (Fig. 5), although no increase in fluorescence was seen after incubation at reduced temperature. Our results demonstrate that a functional TAP complex influences the cell surface expression of free K^b and D^b molecules, from which we infer that free heavy chains can bind peptide in living cells.

Our results provide a biochemical basis for the ability of K^b-restricted CD8$^+$ T cells to interact functionally with H-2^b β_2m$^{-/-}$ cells. One model system for studying positive selection of immature thymocytes in vitro uses FTOC prepared from β_2m$^{-/-}$ mice (41). Recent work has demonstrated that functional CD8$^+$ T cells expressing a transgenic K^b- or D^b-restricted TCR can be generated in β_2m$^{-/-}$ FTOC upon addition of the appropriate peptide and β_2m (13, 42). Our data suggest that a proportion of K^b and D^b molecules at the surface of β_2m$^{-/-}$ cells may contain endogenously bound peptides, based on the requirement for TAP-dependent peptides in the intracellular transport of free K^b and D^b heavy chains. In view of the widely different concentrations of peptide required to rescue emergence of a lymphocytic choriomeningitis virus peptide–specific D^b-restricted TCR in β_2m$^{-/-}$ (42) and in TAP1$^{-/-}$ FTOC (43), it is possible that the presence of TAP-dependent peptides bound to D^b in the former, but not the latter, situation may result in different threshold concentrations of peptide required for key selection events.

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