Brief Definitive Report

Increase in Positive Selection of CD8+ T Cells in TAP1-Mutant Mice by Human β_2-Microglobulin Transgene

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Summary

Mice harboring a deletion of the gene encoding the transporter associated with antigen presentation-1 (TAP1) are impaired in providing major histocompatibility complex (MHC) class I molecules with peptides of cytosolic origin and lack stable MHC class I cell surface expression. They consequently have a strongly reduced number of CD8+ T cells. To examine whether selection of CD8+ T cells is dependent on TAP-dependent peptides, we partially restored MHC class I cell surface expression in TAP1-deficient mice by introduction of human β_2-microglobulin. We show that selection of functional CD8+ T cells can be augmented in vivo in the absence of TAP1-dependent peptides.

CD8+ T lymphocytes are positively selected by MHC class I molecules to ensure self-restriction, a process that requires proper surface expression of MHC class I molecules (1). MHC class I molecules present peptides, derived mainly from cytosolic proteins, to CD8+ T cells. Most MHC class I molecules rely on these peptides, as provided by the heterodimeric transporter associated with antigen processing (TAP) complex, for efficient expression at the cell surface (2). Mice in which the gene encoding the TAP1 subunit is deleted have strongly reduced MHC class I cell surface expression and are impaired in positive selection of CD8+ T cells (3). However, HLA-A2 can be expressed at intermediate levels on the cell surface of TAP-deficient cells, due to its ability to bind signal sequence–derived peptides, a TAP-independent source of peptides (4). We crossed mice transgenic for HLA-A2 and human β_2-microglobulin (hβ_2m) (TAP1+βA2) onto a TAP1-deficient background to examine whether TAP-dependent peptides are essential for development of CD8+ T cells, or whether TAP-independent peptides suffice. We also crossed mice transgenic for HLA-B27 and hβ_2m (TAP1+βB27) with TAP1−-mutant mice. HLA-B27 is inefficiently expressed at the cell surface of TAP-mutant cells (5), and the TAP1−βB27 mice were intended as controls. However, whereas only HLA-A2 was expressed at the cell surface in a TAP1-deficient background, TAP1−βA2 and TAP1−βB27 mice showed a similar increase in percentage of CD8+ T cells compared with TAP1− mice. Rescue of CD8+ T cells is therefore independent of the ability of the transgenic human MHC class I molecules to bind signal sequence–derived peptides and must be due to a feature shared by the transgenic animals, to wit, the presence of hβ_2m.

Materials and Methods

Mice. TAP1-deficient mice have been described previously (3). HLA-A2/hβ_2m– and HLA-B27/hβ_2m–transgenic mice have been described (6). Mice were kept in the animal facilities of the Massachusetts Institute of Technology. Mice 6–10 wk of age were used in all experiments.

Flow Cytometric Analysis. The following monoclonal antibodies and reagents were used: PA2.1 (HLA-A2) (a gift from Dr. H. N. Eisen, Massachusetts Institute of Technology, Cambridge, MA); ME1 (HLA-B27) (a gift from Dr. J. L. Strominger, Harvard University, Cambridge, MA); Y3 (K^b, α_1 + α_2) (American Type Culture Collection, Rockville, MD) and B22-249.R1 (D^b, α_1) (American Type Culture Collection); PE-coupled anti-CD4, FITC-coupled anti CD8-α, biotin-coupled anti-Vβ antibodies and PE-coupled streptavidin (PharMingen, San Diego, CA); PE-coupled rat anti-mouse Ly-5 (B220) antibody (CALTAG Labs, San Francisco, CA); FITC-labeled goat anti-mouse IgG antiserum (Southern Biotechnologies Associates, Birmingham, AL). Single-cell suspensions from spleen, lymph nodes, and thymus were prepared, stained, and ana-

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lyzed on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) as described previously (3). Blood was stained for CD8-expressing cells with the FITC-coupled anti-CD8 antibody. Red blood cells were subsequently lysed with FACS® lysis solution (Becton Dickinson & Co.), and lymphocytes were analyzed on a FACScan®.

Metabolic Labeling and Immunoprecipitations. Spleen cells were cultured for 2 d in DME containing 10% FCS and 2.5 μg/ml Con A. Cells were incubated for 30 min in methionine-free RPMI medium and labeled with [^{35}S]L-methionine/cysteine (protein labeling mix; DuPont/NEN, Boston, MA) for the indicated time and, where appropriate, chased in presence of 1 mM cold L-meth-

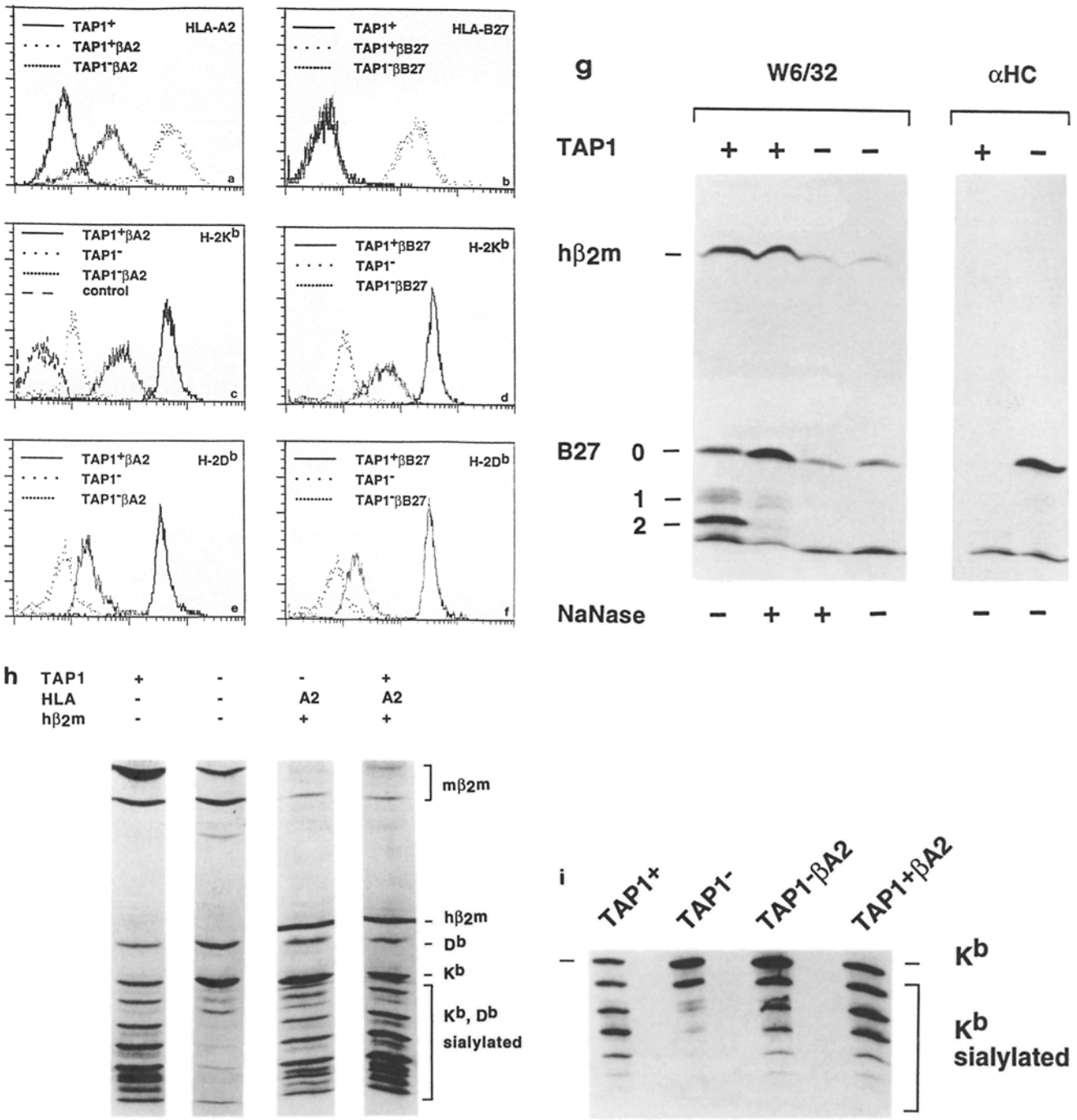

Figure 1. Cell surface expression and intracellular transport of MHC class I molecules in TAP1$^-$βA2 and TAP1$^-$βB27 mice. (*a–f*) Flow cytometric analysis of Ly5$^-$ splenocytes of indicated mice with monoclonal antibodies against (*a*) HLA-A2, (*b*) HLA-B27, (*c* and *d*) H-2K^b, and (*e* and *f*) H-2D^b. (*g*) Metabolic labeling of splenocytes from TAP1$^+$βB27 and TAP1$^-$βB27 mice. Lysates were prepared and B27 complexes were immunoprecipitated with mAb W6/32 and free B27 heavy chains with a rabbit antiserum raised against free human heavy chains. Immunoprecipitates were analyzed on a 1D-IEF gel. (*h*) Pulse–chase analysis on splenocytes from TAP1$^+$βA2, TAP1$^-$βA2, TAP1$^+$, and TAP1$^-$ mice. Cells were labeled for 15 min and chased for 2 h. H-2^b complexes were immunoprecipitated with a conformation-dependent rabbit antiserum raised against purified H-2^b molecules and analyzed on 1D-IEF gels. (*i*) Steady-state distribution of K^b molecules in thymic capsule extracts from TAP1$^+$βA2, TAP1$^-$βA2, TAP1$^+$, and TAP1$^-$ mice.

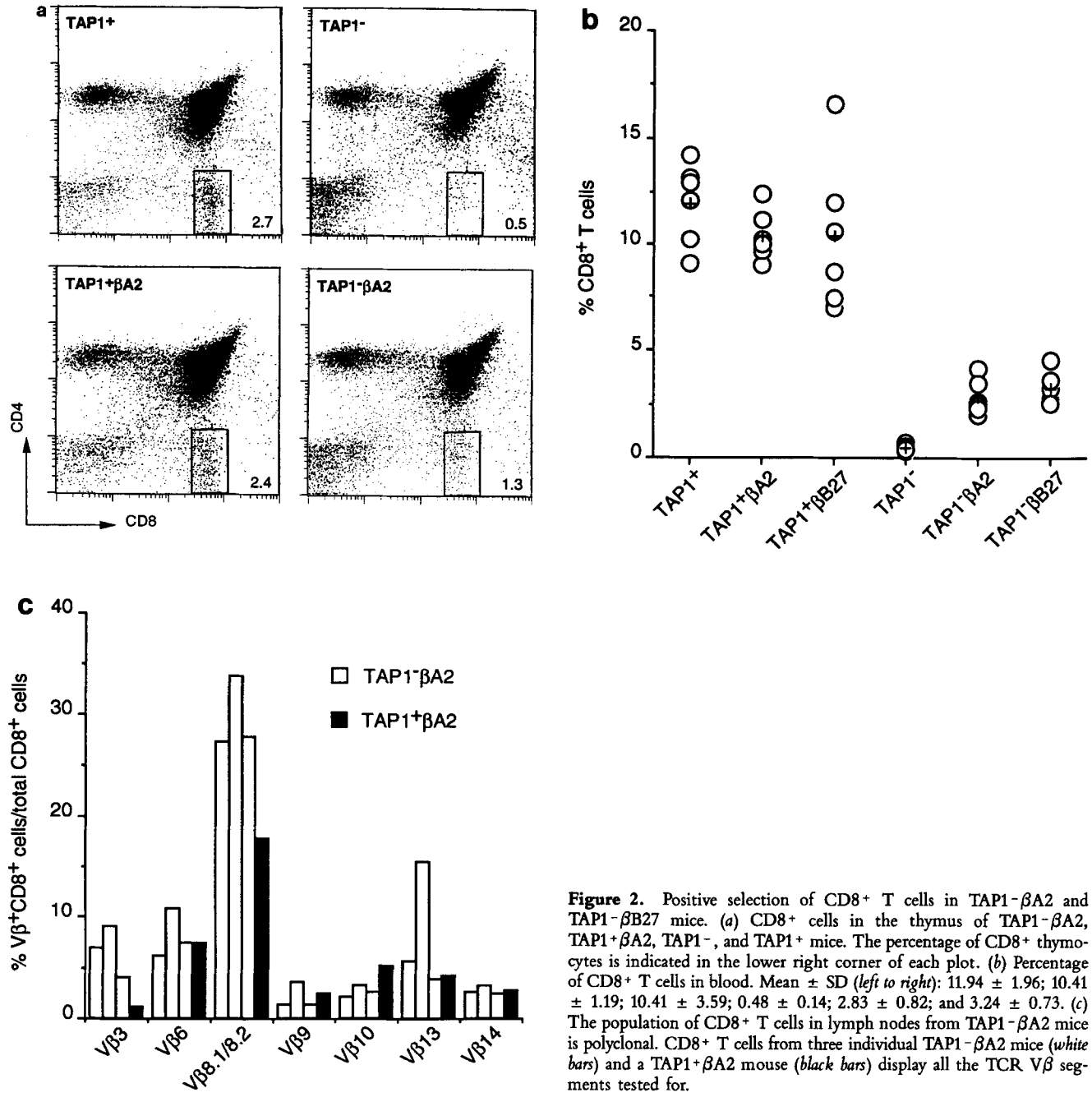

Figure 2. Positive selection of CD8+ T cells in TAP1-βA2 and TAP1-βB27 mice. (*a*) CD8+ cells in the thymus of TAP1-βA2, TAP1+βA2, TAP1-, and TAP1+ mice. The percentage of CD8+ thymocytes is indicated in the lower right corner of each plot. (*b*) Percentage of CD8+ T cells in blood. Mean ± SD (*left to right*): 11.94 ± 1.96; 10.41 ± 1.19; 10.41 ± 3.59; 0.48 ± 0.14; 2.83 ± 0.82; and 3.24 ± 0.73. (*c*) The population of CD8+ T cells in lymph nodes from TAP1-βA2 mice is polyclonal. CD8+ T cells from three individual TAP1-βA2 mice (*white bars*) and a TAP1+βA2 mouse (*black bars*) display all the TCR Vβ segments tested for.

ionine and L-cysteine. Cells were lysed in NP-40 lysis mix (0.5% NP-40, 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.1 mM PMSF). Immunoprecipitations, neuraminidase digestion, and one-dimensional isoelectric focusing (1D-IEF) were performed as previously described (7).

Steady State Distribution of K^b Molecules in the Thymus. Thymuses were isolated from 6–8-wk-old mice. Thymocytes were removed by gently squeezing the thymic lobes with forceps and rinsing with PBS. The remaining capsule was macerated and dissolved in IEF sample buffer by repeated shearing through a 25G1$^1/_2$ needle. Samples were separated on 1D-IEF and blotted to nitrocellulose paper. The blot was incubated with a rabbit antiserum raised against a peptide derived from the cytoplasmic tail of K^b (αp8) followed by a horseradish peroxidase–coupled goat anti-rabbit antibody (Amersham, Arlington Heights, IL). Bound antibody was detected by a chemiluminescence detection kit (Amersham) and exposure to films (X-OMAT AR; Eastman-Kodak Co., Rochester, NY).

Stability Assay. Freshly isolated spleen cells were labeled by lactoperoxidase-catalyzed iodination (see reference 18). Cells were lysed in 2 ml NP-40 lysis mix. Lysates were divided into four equal parts and were incubated for 15 min on ice after addition of 0.5 ml lysis mix with or without 60 μM YAPGNFPAL peptide. Samples were kept on ice or incubated at 39°C for 45 min. Lysates were precleared twice, followed by immunoprecipitation of $H\text{-}2^b$ com-

plexes with a conformation-dependent anti-H-2^b serum (a gift from Dr. S. Nathenson, Albert Einstein College of Medicine, Bronx, NY). Immunoprecipitates were analyzed by 12.5% SDS-PAGE.

Peptide-binding Assay. YAPGNFPAL peptide was labeled by chloramine T-catalyzed iodination (see reference 11). 10^6 splenocytes were incubated in PBN buffer (PBS + 1% BSA + 0.01% NaN_3) with the indicated concentrations of ^{125}I-YAPGNFPAL for 1 h at 23°C. Cells were lysed in NP-40 lysis mix containing 100 μM cold FAPGNYPAL. Lysates were precleared once, and K^b molecules were precipitated with the αp8 serum. Immunoprecipitates were counted in a γ counter. Values are the mean of triplicates.

Results.

Spleen cells from TAP1$^-$$\beta$A2 mice were stained with an mAB directed against HLA-A2. They express an intermediate level of HLA-A2 on the cell surface (Fig. 1 *a*) compared with cells from TAP1$^+$$\beta$A2 mice. This is in agreement with observations on TAP-deficient cell lines (8). The cell surface expression of HLA-A2 is paralleled by an approximately twofold higher percentage of CD8$^+$ cells in the thymus (Fig. 2 *a*) and an approximately sixfold higher percentage of CD8$^+$ T cells in blood (Fig. 2 *b*) in TAP1$^-$$\beta$A2 mice, as compared with TAP1$^-$ mice.

HLA-B27 cannot be detected either at the cell surface in TAP1-deficient animals, in contrast to HLA-A2 (Fig. 1 *b*). A 3-h metabolic labeling of TAP1$^-$$\beta$B27 spleen cells followed by immunoprecipitation with the conformation-dependent antibody W6/32 and analysis of the immunoprecipitates on a 1D-IEF gel shows that a substantial fraction of the HLA-B27 molecules fail to assemble with β_2m (Fig. 1 *g*). Parallel immunoprecipitation with an antiserum raised against denatured free heavy chains reveals that most B27 molecules are present as free heavy chains (Fig. 1 *g*). The few complexes that are present do not carry sialic acids, the acquisition of which is indicative of proper intracellular transport. These data underscore the reliance of HLA-B27 on TAP for a suitable source of peptides required for assembly and surface expression. Nonetheless, the TAP1$^-$$\beta$B27 mice show a similar increase in the percentage of CD8$^+$ T cells in the periphery, as do the TAP1$^-$$\beta$A2 mice (Fig. 2 *b*).

By examination of the surface expression of H-2K^b and D^b, this paradox may be satisfactorily explained. Surface expression of H-2K^b and D^b class I molecules in a murine TAP2-deficient cell line can be increased by transfection of this cell line with hβ_2m (5). Indeed, K^b shows a fivefold and D^b a twofold increase in cell surface expression on spleen cells from both TAP1$^-$$\beta$A2 and TAP1$^-$$\beta$B27 mice compared with TAP1$^-$ mice (Fig. 1, *c–f*). Pulse–chase analysis shows that these K^b and D^b heavy chains preferentially associate with hβ_2m and are transported to the cell surface, as judged also by acquisition of sialic acids (Fig. 1 *h*).

Relative levels of MHC class I surface expression in the thymus were determined at steady state. Thymic lobes were depleted of thymocytes, and extracts of the remaining thymic capsules were resolved on 1D-IEF and analyzed by immunoblotting. In TAP1$^-$ animals, the majority of K^b heavy chains remains unmodified (Fig. 1 *i*), but in TAP1$^-$$\beta$A2 animals (and TAP1$^-$$\beta$B27 animals, data not shown) modification of K^b is observed, indicating that the K^b molecules have

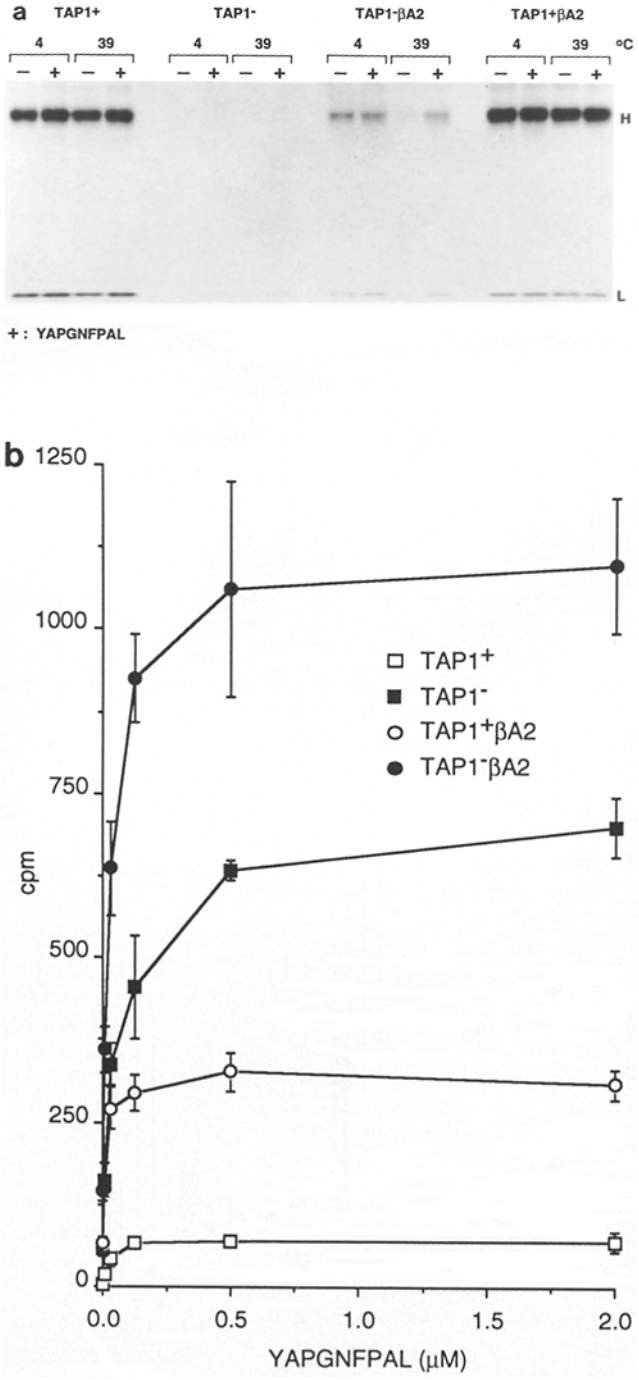

Figure 3. Stability and peptide binding capacity of MHC class I molecules. (*a*) Stabilization of H-2^b molecules by the YAPGNFPAL peptide. Cell lysates of cell surface–labeled spleen cells from the indicated mice were preincubated with (+) or without (−) 30 μM YAPGNFPAL and subsequently kept at 4°C or 39°C. H-2^b molecules were then immunoprecipitated with the conformation-dependent anti–H-2^b serum and analyzed by SDS-PAGE. (*b*) Relative ^{125}I-YAPGNFPAL peptide-binding capacity of K^b molecules on the cell surface of TAP1$^-$$\beta$A2, TAP1$^+$$\beta$A2, TAP1$^-$, and TAP1$^+$ cells. YAPGNFPAL peptide was used for iodination instead of FAPGNYPAL to prevent rapid dissociation of bound peptide from the MHC class I complex caused by iodination of the tyrosine anchor residue (14).

traversed the *trans*-Golgi network. Thus both in the periphery and in the thymus, surface expression of endogenous MHC class I molecules is increased in the presence of hβ_2m. This increase in MHC expression may explain selection of CD8$^+$ T cells in the TAP1$^-\beta$A2 and TAP1$^-\beta$B27 mice.

We examined polyclonality of the CD8$^+$ T cell population present in TAP1$^-\beta$A2 mice. Cell suspensions, made from lymph nodes of TAP1$^-\beta$A2 and TAP1$^+\beta$A2 mice, were stained with antibodies against CD8 and different TCR Vβ chains. Of all the Vβ chains tested, those used by CD8$^+$ T cells in TAP1$^+\beta$A2 mice are also used by CD8$^+$ T cells in TAP1$^-\beta$A2 mice, indicating that the CD8$^+$ T cell population in TAP1$^-\beta$A2 mice is polyclonal (Fig. 2 *c*). These CD8$^+$ T cells are able to respond in a primary mixed lymphocyte reaction. After 5 d of culture, strong CD8$^+$ T cell–dependent cytotoxicity against H-2^d targets is observed, similar to that in TAP1$^+\beta$A2 mice (data not shown).

MHC molecules from TAP-deficient cells (9–11) and mice (3) have been proposed to be devoid of peptide based on their failure to be expressed efficiently at the cell surface, their thermolability, and their increased peptide-binding capacity. Do MHC class I molecules in TAP1$^-\beta$A2 mice display similar properties? Detergent lysates of cell surface–iodinated spleen cells were incubated at 4°C or 39°C in the presence or absence of the 9-mer peptide YAPGNFPAL, a variant of the Sendai virus peptide FAPGNYPAL (12, 13) that contains the anchor residues for both K^b and D^b. H-2^b class I complexes were then immunoprecipitated with a conformation-specific anti H-2^b serum. Labeled MHC class I complexes are absent from lysates of TAP1$^-$ cells, but they are detected in lysates from TAP1$^-\beta$A2 cells (Fig. 3 *a*) in amounts in accordance with the data obtained by flow cytometric analysis (Fig. 1, *c* and *e*). Exposure of lysates of TAP1$^-\beta$A2 cells to 39°C results in a strong decrease in reactivity with the conformation-specific anti H-2^b antiserum, but addition of YAPGNFPAL peptide prevents thermal unfolding of the H-2^b molecules (Fig. 3 *a*). No such loss of immunoreactive material is observed in extracts from TAP1$^+$ and TAP1$^+\beta$A2 cells.

To determine the pool size of K^b molecules on the cell surface that are available for binding class I peptides, a peptide-binding assay was performed on freshly isolated splenocytes by use of radiolabeled ^{125}I-YAPGNFPAL. K^b molecules on splenocytes from both TAP1$^-$ and TAP1$^-\beta$A2 mice bind considerably more ^{125}I-YAPGNFPAL than K^b molecules on TAP1$^+$ and TAP1$^+\beta$A2 cells (Fig. 3 *b*), despite lower levels of K^b cell surface expression (Fig. 1 *c*). An approximately twofold difference in binding capacity between TAP1$^-\beta$A2 and TAP1$^-$ cells is observed, whereas TAP1$^-\beta$A2 cells express approximately fivefold more K^b molecules on the cell surface. A significant proportion of K^b molecules on TAP1$^-\beta$A2 cells may have bound peptides from a TAP-independent source (15). These peptides presumably bind with a lower affinity than TAP-dependent peptides, since they fail to stabilize H-2^b complexes in vitro (Fig. 3 *a*).

Discussion

In both β_2m$^-$ and TAP1$^-$ mice, small numbers of CD8$^+$ T cells are present that can be expanded in vivo and vitro (16–19). Our data show that selection of CD8$^+$ T cells in TAP1$^-$ mice can be augmented in vivo by increasing cell surface expression of H-2K^b and D^b through heterodimerization with hβ_2m. In vivo selection of CD8$^+$ T cells is therefore not strictly dependent on peptides of cytosolic origin and can be mediated by MHC class I molecules that are either devoid of peptide, have bound peptides from a TAP-independent source, or both. The pool of CD8$^+$ T cells selected by these molecules is polyclonal and alloreactive.

In fetal thymic organ cultures (FTOCs), an in vitro model for thymic selection, peptides contribute to the specificity of positive selection (20–23). In FTOCs derived from β_2m$^-$ mice, cell surface expression of MHC class I molecules is not detectable, and structural similarity between the selecting peptide and the nominal antigen is required for positive selection of a monoclonal T cell population bearing a K^b-restricted, ovalbumin-specific TCR (22). Selection in TAP1$^-$ FTOCs of a D^b-restricted lymphocytic choriomeningitis virus peptide–specific T cell can be accomplished by the nominal antigen at low peptide concentrations (30 μM) (23). However, at a 10-fold higher peptide concentration, and therefore at much higher MHC class I density (21), the same T cell can be selected by a D^b-binding peptide (influenza NP366-374) structurally unrelated to the nominal antigen. At physiological densities of MHC class I/peptide complexes, selection of a given CD8$^+$ T cell may be less dependent on a specific peptide and may be accomplished by MHC class I molecules bearing a heterogeneous set of peptides not necessarily related to the nominal antigen other than by their ability to bind to the restriction element in question.

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