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TAP1 Mutant Mice Are Deficient in Antigen Presentation, Surface Class I Molecules, and CD4$^-$8$^+$ T Cells

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Summary

The transporter associated with the antigen processing 1 (*TAP1*) gene encodes a subunit for a transporter, presumed to be involved in the delivery of peptides across the endoplasmic reticulum membrane to class I molecules. We have generated mice with a disrupted *TAP1* gene using embryonic stem cell technology. TAP1-deficient mice are defective in the stable assembly and intracellular transport of class I molecules and consequently show severely reduced levels of surface class I molecules. These properties are strikingly similar to those described for the *TAP2* mutant cell line RMA-S. Cells from the TAP1-deficient mice are unable to present cytosolic antigens to class I–restricted cytotoxic T cells. As predicted from the near absence of class I surface expression, TAP1-deficient mice lack CD4$^-$8$^+$ T cells.

Introduction

Class I major histocompatibility complex (MHC) antigens are composed of polymorphic heavy chains noncovalently associated with β_2-microglobulin (Bjorkman et al., 1987). They present peptides, usually 8–9 amino acids long, to CD8$^+$ T lymphocytes (for review see van Bleek and Nathenson, 1992). Such peptides are thought to arise from cleavage of proteins in the cytosol (Moore et al., 1988; Yewdell et al., 1988) and are transported by a signal sequence–independent mechanism into the endoplasmic reticulum (ER), where assembly with class I molecules is thought to take place (Townsend and Bodmer, 1989).

Mutant cell lines with defects in the presentation of antigenic peptides to T cells have been identified. Prototypes of these are the mouse T lymphoma cell line RMA-S (Ljunggren and Kärre, 1985; Kärre et al., 1986) and the human B lymphoblastoid cell lines 721.174 (and its derivative T2) and 721.134 (Kavathas et al., 1980; DeMars et al., 1984). These cell lines, when infected with virus, fail to be efficiently recognized by antigen-specific cytotoxic T lymphocytes (CTLs) (Townsend et al., 1989a). They are deficient in the cell surface expression of class I molecules, although the heavy and light chains of class I molecules are synthesized normally and complex formation between class I heavy chains and β_2-microglobulin does occur (Ljunggren et al., 1990a; Baas et al., 1992). Surface expression could be restored, at least in part, by culture at reduced temperature (Ljunggren et al., 1990a) or by incubation with presentable peptide (Townsend et al., 1989a). It was suggested that these mutant cell lines have a defect in the transport of peptides from the cytosol into the ER and that class I molecules require peptide for stable surface expression at 37°C (Townsend et al., 1989b).

The genetic elements responsible for the defect in the human cell line 721.174 were mapped to the MHC class II region (DeMars et al., 1985; Cerundolo et al., 1990; Hosken and Bevan, 1990). This region contains two genes, now known as *TAP1* and *TAP2* (previous designations are *HAM1/2*, *PSF1/2* or *RING4/11*, and *MTP1/2* in mouse, human, and rat, respectively; for the nomenclature see the World Health Organization Nomenclature Committee, 1992), encoding proteins with structural similarity to ATP-binding transporters (Deverson et al., 1990; Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990). Transfection of *TAP1* cDNA into mutant 721.134 resulted in complete restoration of MHC class I surface expression (Spies and DeMars, 1991). The same cDNA could not correct the defect in 721.174 cells (Spies and DeMars, 1991) since this cell line has a homozygous deletion in the class II region encompassing both *TAP1* and *TAP2* (Spies et al., 1990). The antigen-processing defect in the RMA-S cell line was restored by transfection with the *TAP2* cDNA (Powis et al., 1991; Attaya et al., 1992). TAP1 and TAP2 have been suggested to form a heterodimeric complex to transport peptides across the ER membrane (Deverson et al., 1990; Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990). Three pieces of evidence further support this hypothesis. First, antisera raised against peptides derived from TAP1 amino acid sequences immunoprecipitated both TAP1 and TAP2 from human cells (Kelly et al., 1992; Spies et al., 1992). Second, immunohistochemical analysis showed that the TAP1 immunoreactivity is located in the ER and cis Golgi (Kleijmeer et al., 1992). Third, transfection of *TAP1* and *TAP2* cDNA in the T2 cell line is both necessary and sufficient for rescue of class I surface expression (Momburg et al., 1992).

Mutant cell lines from lymphoid origin have been used to study class I expression in relation to the proposed function of the TAP1–TAP2 transporter. The possible involvement of alternate routes of peptide loading in other cell types such as thymic epithelial cells cannot be studied with these mutant cell lines. We therefore introduced a deletion in the *TAP1* gene of embryonic stem (ES) cells by gene targeting (Capecchi, 1989) and produced mice homozygous for this mutation. Cells from TAP1-deficient mice lack class I cell surface expression and are defective in the intracellular transport and assembly of class I molecules. Cell surface expression can be restored by treatment of the cells with presentable peptides or by culture at reduced temperature to an extent similar to that observed for RMA-S. These cells are unable to present intracellular protein antigens to class I–restricted cytotoxic T cells. *TAP1*$^{-/-}$ mice show a normal distribution of CD4$^+$8$^-$ and CD4$^+$8$^+$ T cells but have no mature CD4$^-$8$^+$ T cells. The availability of a mouse model with a defined defect in the loading of class I molecules with peptides should prove useful to approach

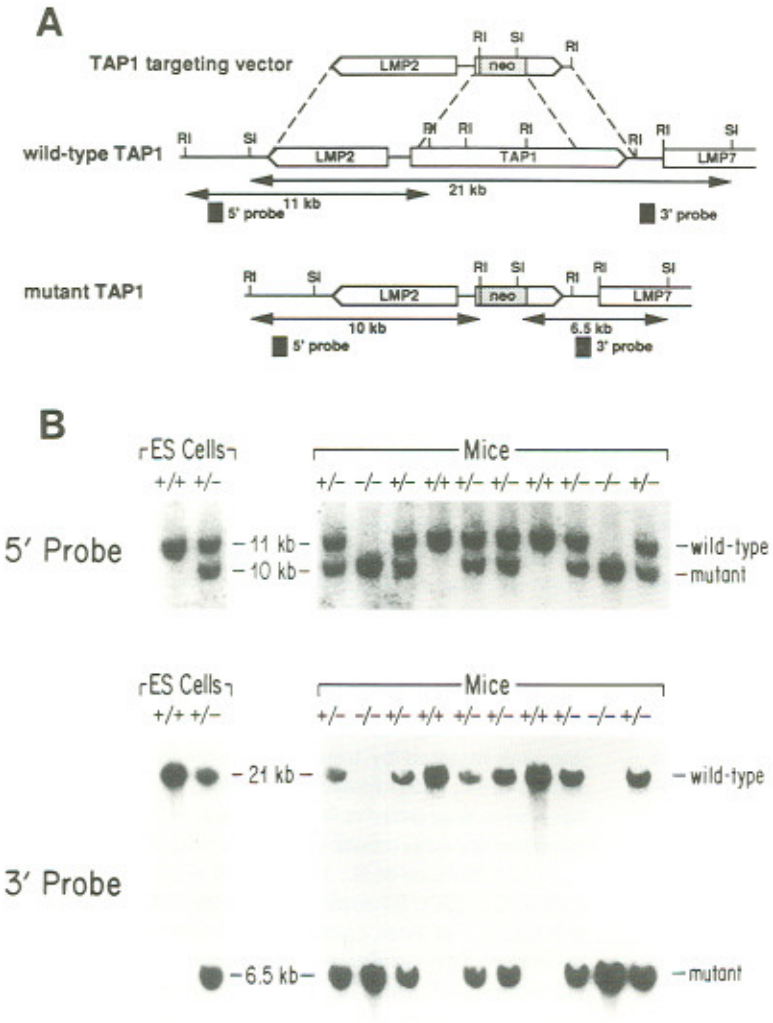

Figure 1. Production of *TAP1*$^{-/-}$ Mice

(A) The *TAP1* locus and targeting construct. The *TAP1* gene is shown, along with two other genes in the class II region (*LMP2* and *LMP7*), encoding cytosolic proteasome components (for review see Driscoll and Finley, 1992). The exon–intron structure of these genes has not been described yet. A 7 kb fragment from the *TAP1* gene was deleted and replaced with a *neo* gene. *TAP1* 5′ and 3′ flanking probes used for screening of ES cell clones and mice are indicated, together with the expected size of hybridizing restriction fragments in wild-type and mutant *TAP1* alleles (see [B]). Abbreviations for restriction sites: RI, EcoRI; SI, ScaI.

(B) Southern blot analysis of representative ES cell clones and tail biopsies. Genomic DNA was isolated from an ES cell clone with a mutated *TAP1* allele and a wild-type control (left) and from a litter of ten mice from a heterozygous intercross (right). DNA was digested with EcoRI (top) or ScaI (bottom) and hybridized with the 5′ and 3′ flanking probes (see [A]), respectively. Wild-type and targeted alleles are indicated. Two mice in the litter are homozygous for the mutation.

the involvement of self-peptide–class I complexes in selection of the T cell repertoire.

Results

Generation of TAP1-Deficient Mice

The *TAP1* targeting vector (Figure 1A) was constructed from genomic clones isolated from a C57BL/6 cosmid library. Upon homologous recombination, this construct would remove a 7 kb region from the *TAP1* gene, encompassing about 80% of the protein-encoding region. The deleted region was replaced by a neomycin resistance gene (*neo*) controlled by the phosphoglycerate kinase 1 (*PGK1*) promoter.

The targeting construct was transfected into ES cells of the D3 line (derived from the strain 129/Sv) and subjected to G418 selection. Resistant clones were screened for the desired homologous recombination event by Southern blotting. Blots were hybridized with 5′ and 3′ flanking probes (Figure 1A). Of 576 clones analyzed, 9 carried the mutation. The result for one targeted clone and a wild-type control is shown as an example in Figure 1B. Four clones were injected into C57BL/6 blastocysts, and the embryos were reimplanted into foster mothers. All injected clones gave rise to chimeric mice, and these were mated with C57BL/6 mice. Chimeras from three of the injected clones were shown to generate offspring heterozygous for the mutation. Finally, (129/Sv × B6) F1 heterozygotes were intercrossed to produce *TAP1*$^{-/-}$ homozygotes (Figure 1B).

The F2 animals were raised under germ-free conditions and appeared healthy.

TAP1-Deficient Mice Lack Class I Cell Surface Expression

We evaluated the cell surface expression of class I molecules on spleen cells from the *TAP1* mutant mice by cytofluorimetry using a panel of class I–specific monoclonal antibodies. There is no detectable staining of cells from the mutant mice with the anti-D^b antibody B22-249.R1 (α_1-specific) or the anti-K^b antibody AF6-88.5, as compared with the staining of BALB/c spleen cells (Figure 2). Only background staining was seen with a variety of other antibodies: H141-31-10 (D^b and α_2 specific), B8-24-3 (K^b and α_1 specific), Y-3 (K^b and $\alpha_1+\alpha_2$ specific), 20-8-4S (K^b and

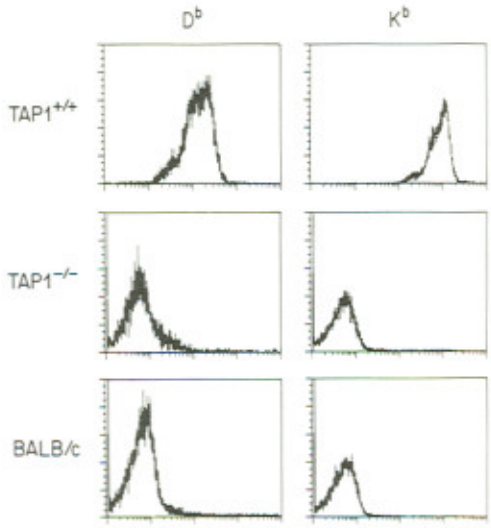

Figure 2. Cell Surface Expression of Class I Molecules

Spleen cells isolated from wild-type (*TAP1*$^{+/+}$), mutant (*TAP1*$^{-/-}$), or BALB/c (H-2^d) mice were incubated with the anti-H-2 D^b antibody B22-249.R1 (α_1 specific) or the anti-H-2 K^b antibody AF6-88.5 followed by goat anti-mouse FITC-labeled immunoglobulin G and analyzed by FACS. Negative controls (minus) were stained with the second antibody only.

D^b), and 28-8-6S (K^b, D^b, α_1, and/or α_2 specific) (data not shown). Surface expression on wild-type spleen cells and on cells from heterozygous mice was indistinguishable (data not shown).

Can *TAP1*$^{-/-}$ cells serve as targets for anti-H-2^b CTLs generated in a conventional mixed lymphocyte reaction? About 25-fold more CTLs are required to lyse mutant cells compared with wild-type cells (Figure 3A). Similarly, RMA-S cells, which are deficient in TAP2, needed about 25 times more CTLs to be lysed. The observed residual killing for both *TAP1*$^{-/-}$ and RMA-S cells can be explained in several ways. First, the existence of CTLs specific for empty class I molecules has been hypothesized (Aosai et al., 1991) and such empty class I molecules are likely to be present at the cell surface in small amounts. Peptide and/or the bovine β_2-microglobulin from the serum-containing medium, or peptide, β_2-microglobulin, or both released from dead cells could stabilize surface expression of class I on these cells. Finally, residual CTL activity might be directed against nonclass I antigens.

When mutant cells were used as stimulators to prepare anti-H-2^b CTLs, similar residual reactivity was observed (Figure 3B).

Defective Intracellular Transport and Assembly of Class I Molecules

The extent of intracellular transport of class I molecules can be estimated from the presence and rate of glycan modifications (sialylation) on the class I heavy chain. Addition of sialic acid takes place in the trans-Golgi reticulum and results in a more acidic isoelectric point of the class I heavy chains.

In pulse–chase experiments on wild-type spleen cells and RMA-S cells, both K^b and D^b molecules rapidly acquire sialic acids, a process essentially complete after 1 hr of chase (Figure 4). For *TAP1* mutant cells and RMA-S cells, only low levels of sialylated forms can be detected, indicating that the delivery of both K^b and D^b molecules to the trans Golgi is impaired in these cells. Immunoprecipitation with an antiserum directed against the cytoplasmic tail of H-2 K^b gives a very similar picture (Figure 5A), indicating that this inhibition of transport applies to all K^b molecules, regardless of their conformation and association with β_2-microglobulin.

It has been proposed that the lack of class I transport in RMA-S cells is caused by the instability of empty class I molecules that undergo an irreversible conformational change at 37°C. Such empty and incorrectly folded class I molecules would not be transported further and rerouted or degraded (Ljunggren et al., 1990a). Recycling of class I molecules between the ER and the cis-Golgi complex has been reported for other cell lines deficient in class I expression (Hsu et al., 1991). For RMA-S cells, transport of class I molecules can be restored, at least partially, by culture at reduced temperature (Ljunggren et al., 1990a; see Figure 4). To investigate whether this is also the case for cells from the *TAP1*$^{-/-}$ mice, pulse–chase experiments were performed at 26°C (see Figure 4). For wild-type cells, glycan modifications were slower compared with chase at 37°C. For the mutant cells, the disappearance of immunoreactive class I molecules was drastically decreased, and, in addition, extensive sialylation was observed. These results demonstrate that, similar to RMA-S cells, intracellular transport in *TAP1*$^{-/-}$ cells can be partially restored by incubation at 26°C.

To address stability of class I molecules in *TAP1* mutant cells directly, detergent lysates from metabolically labeled cells were incubated at different temperatures (4°C, 15°C, 26°C, and 37°C) (Figure 5A). There was a strong reduction in the number of anti-H-2^b immunoprecipitable class I molecules with increasing temperature, whereas the number of immunoprecipitable class I molecules in control wild-type extracts was unchanged. H-2 K^b chains in lysates from *TAP1* mutant cells, regardless of the temperature to which they are exposed, are not destroyed by proteolysis, as demonstrated by the presence of K^b heavy chains revealed by immunoprecipitation with the conformation-independent anti-K^b exon 8 serum. In conclusion, these results indicate that the class I molecules in *TAP1* mutant cells are intrinsically unstable.

Stabilization of Class I Cell Surface Expression and Assembly by Peptides or Low Temperature

Cell surface expression and stable assembly of class I molecules in RMA-S cells can be enhanced by treatment of cells with synthetic peptide or by incubation at reduced temperature (Townsend et al., 1989a, 1990; Ljunggren et al., 1990a; Schumacher et al., 1990; Rock et al., 1991).

We cultured spleen cells from *TAP1* mutant mice with different concentrations of synthetic peptides in the presence of bovine β_2-microglobulin. Surface expression was judged by antibody staining and fluorescence-activated cell sorting (FACS) analysis (Figure 6). The vesicular sto-

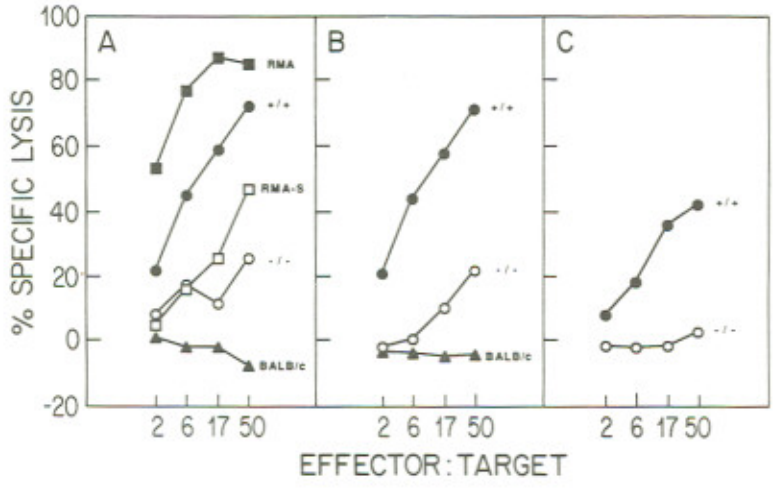

Figure 3. T Cell–Mediated Cytotoxicity Assays with Cells from *TAP1* Mutant Mice

(A) *TAP1* mutant spleen cells as targets for alloreactive CTLs. Alloreactive CTLs were generated in a bulk mixed lymphocyte culture of BALB/c (H-2^d) spleen cells with irradiated wild-type (H-2^b) splenocytes. This culture was tested for lysis of mutant (open circle), wild-type (closed circle), or BALB/c (closed triangle) concanavalin A–induced blast cells, using ^{51}Cr-release assays. In comparison, RMA-S (open square) and RMA (closed square) targets were used.

(B) *TAP1* mutant spleen cells as inducers of alloreactive CTLs. BALB/c (H-2^d) spleen cells were stimulated with spleen cells from mutant (open circle), wild-type (closed circle), or BALB/c (closed triangle) mice. Concanavalin A–induced wild-type (H-2^b) blasts were used as targets.

(C) Allogeneic spleen cells fail to induce alloreactive CTLs in mixed lymphocyte cultures with *TAP1*$^{-/-}$ spleen cells. Spleen cells from −/− F2 animals (open circle) or +/+ (closed circle) controls were stimulated with irradiated BALB/c (H-2^d) spleen cells, and cultures were tested for lysis of BALB/c concanavalin A–induced blast cells.

matitis virus (VSV) nucleoprotein (NP) peptide (amino acids 52–59), the Sendai virus (SV) NP peptide (324–332), and the ovalbumin (OVA) peptide (257–264) are recognized by CTLs in an H-2 K^b-restricted fashion, whereas the influenza virus (IF) NP peptide (366–374) is recognized by D^b-restricted CTLs. In addition, SV-NP(324–332) was shown to stabilize D^b (Deres et al., 1992). Figure 6 shows that VSV-NP(52–59) stabilizes the surface expression of K^b, SV-NP(324–332) induces an increase in both D^b and K^b surface expression, IF-NP(366–374) induces D^b expression, and OVA(257–264) induces K^b and, to a lesser extent, D^b molecules (Figure 6). The binding of OVA(257–264) to D^b has not been shown before but is perhaps not surprising since its amino acid sequence shows significant homology to the H-2 D^b peptide motif, as deduced from amino acid sequences of self-peptides eluted from D^b molecules (Falk et al., 1991).

Stabilization of class I molecules in detergent extracts was also restored by exposure of lysates from metabolically labeled mutant cells to synthetic peptides (see Figure 5B). Preincubation of lysates with VSV-NP(52–59) made K^b molecules resistant to subsequent treatment at 37°C. Similarly, SV-NP(324–332) stabilized K^b, IF-NP(366–374) stabilized D^b, and OVA(257–264) stabilized K^b molecules. At the peptide concentrations (1–100 nM) used in Figure 5B, we could not detect any significant induction in the

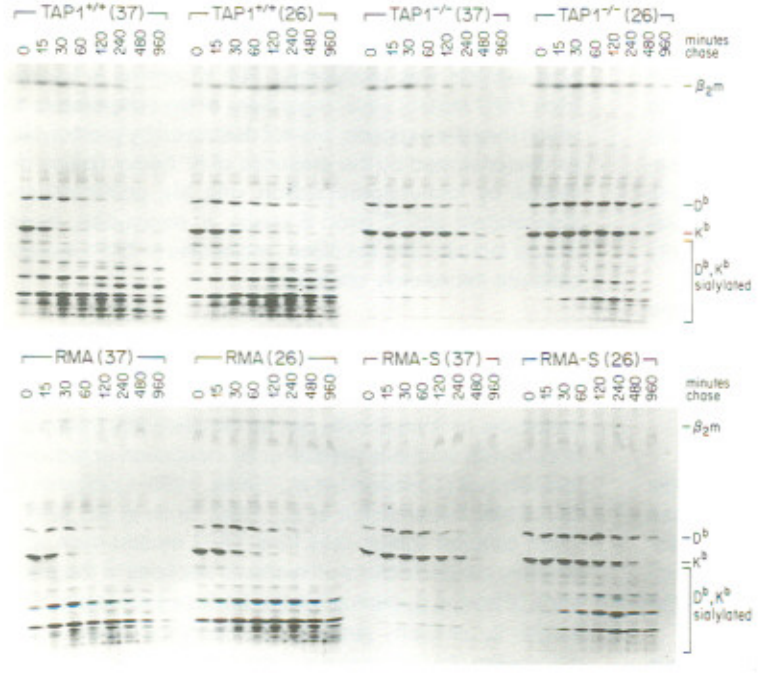

Figure 4. Intracellular Class I Transport

Concanavalin A–induced blast cells from *TAP1* mutant mice or wild-type littermates and RMA-S or RMA cells were metabolically labeled with [^{35}S]methionine for 10 min at 37°C and then chased with cold methionine for the times indicated at either 37°C or 26°C. Class I molecules were immunoprecipitated with an anti-H-2^b serum and analyzed on one-dimensional isoelectrofocusing gels. The focusing positions of β_2-microglobulin and of nonsialylated and sialylated H-2 D^b and H-2 K^b molecules are indicated.

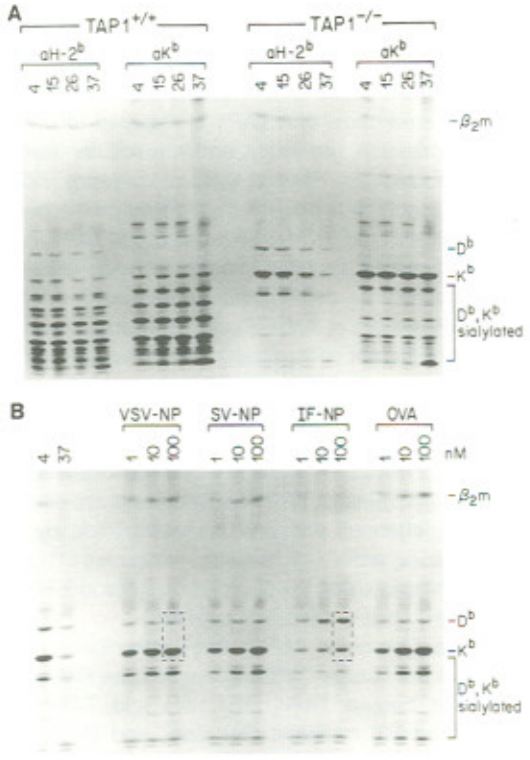

Figure 5. Class I Stability in Detergent Lysates from *TAP1* Mutant Cells

(A) Thermolability of class I molecules in mutant cells. Detergent extracts from mutant (*TAP1*$^{-/-}$) or wild-type (*TAP1*$^{+/+}$) lymphoblasts were incubated for 1 hr at the indicated temperatures, and class I molecules were immunoprecipitated with a conformation-dependent anti-H-2^b serum or a conformation-independent anti-H-2 K^b exon 8 serum. Immunoprecipitates were analyzed by isoelectric focusing and autoradiographed. The focusing positions of β_2-microglobulin and of nonsialylated and sialylated H-2 D^b and H-2 K^b chains are indicated.

(B) Stabilization of class I molecules in detergent lysates from *TAP1*$^{-/-}$ cells by synthetic peptides. Extracts from mutant cells were first treated for 1 hr with the indicated concentrations of synthetic peptides, followed by a 1 hr incubation at 37°C to eliminate empty molecules. Class I molecules were immunoprecipitated with the anti-H-2^b serum and analyzed by isoelectric focusing. At the left, immunoprecipitations are shown from untreated extracts that were incubated for 1 hr at 4°C or 37°C. The ability of VSV-NP to stabilize H-2 K^b and IF-NP to stabilize H-2 D^b chains is apparent from a comparison of the relative intensity of these polypeptides in the boxed areas. By setting the levels of D^b and K^b, measured by densitometry of suitably exposed autoradiographs, for the 4°C samples at 100%, we observed residual levels of 40% and 16.5% for D^b and K^b, respectively, when exposed at 37°C with no peptide added. Maximum stabilization for D^b with VSV, SV, IF, and OVA peptides was 34%, 49%, 137%, and 66%, respectively, and for K^b, 111%, 110%, 27%, and 164%, respectively. For both D^b and K^b, full stabilization by the appropriate peptides was therefore observed.

stability of D^b by SV-NP(324–332) or OVA(257–264), whereas the same peptides were able to induce the cell surface expression of D^b molecules (Figure 6). At higher peptide concentrations (1 μM and higher), stabilization of D^b by SV-NP(324–332) was readily detected (data not shown), but even at very high peptide concentrations (100 μM), we could not detect D^b stabilization by OVA(257–264) (data not shown). The weak binding of these peptides to D^b does not survive immunoprecipitation very well.

Culture of mutant cells at reduced temperature (26°C) similarly increased the cell surface expression of class I molecules (Figure 7). When, after culture at 26°C, the temperature was shifted to 37°C for 1 hr, most of the class I molecules induced at the lower temperature disappeared from the cell surface (Figure 7). This suggests that the class I molecules induced at low temperature on *TAP1*$^{-/-}$ cells lack peptide in their antigen-binding pocket, as was suggested for the RMA-S cell line (Ljunggren et al., 1990a).

Defective Antigen Presentation

The ability of mutant cells to act as targets for killing by an H-2 K^b-restricted, OVA-specific CTL clone (4G3) was compared with wild-type cells. After cytoplasmic loading of OVA by the osmotic lysis of pinosomes, wild-type but not *TAP1* mutant or RMA-S target cells were lysed by the CTL clone (Figure 8). Both wild-type and mutant cells were sensitized for lysis by the CTL clone when exogenous OVA(257–264) peptide was added to the culture.

Absence of CD4$^-$8$^+$ Cytotoxic T Cells

Lymphoid organs and blood in mutant mice were characterized for the presence of different T cell subsets by FACS

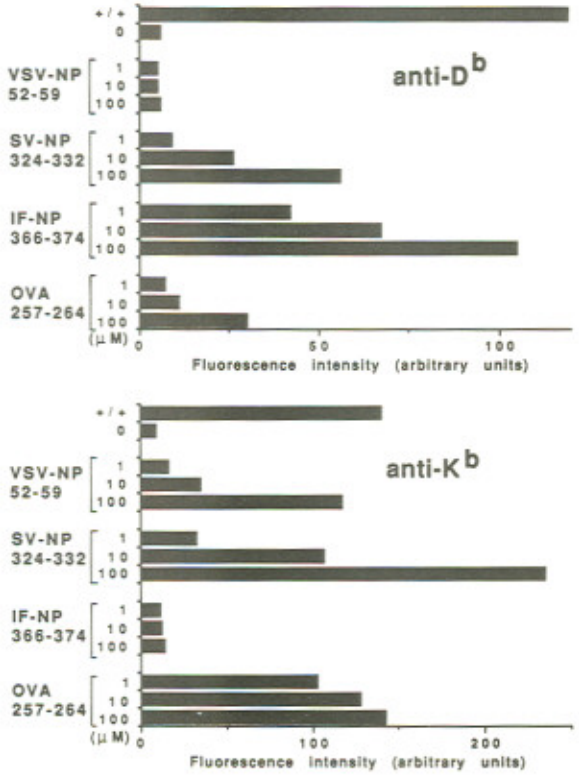

Figure 6. In Vitro Rescue of Class I Expression on *TAP1* Mutant Cells by Synthetic Peptides

Spleen cells from *TAP1* mutant mice were cultured in serum-containing medium with the indicated concentrations of synthetic peptides for 16 hr. Cells were stained with an anti-D^b (B22-249.R1, α_1-specific) or anti-K^b (B8-24-3, α_1-specific) antibody followed by goat anti-mouse FITC-labeled immunoglobulin G and analyzed by FACS. As controls, wild-type cells (+/+) and untreated *TAP1*$^{-/-}$ cells (0) were stained.

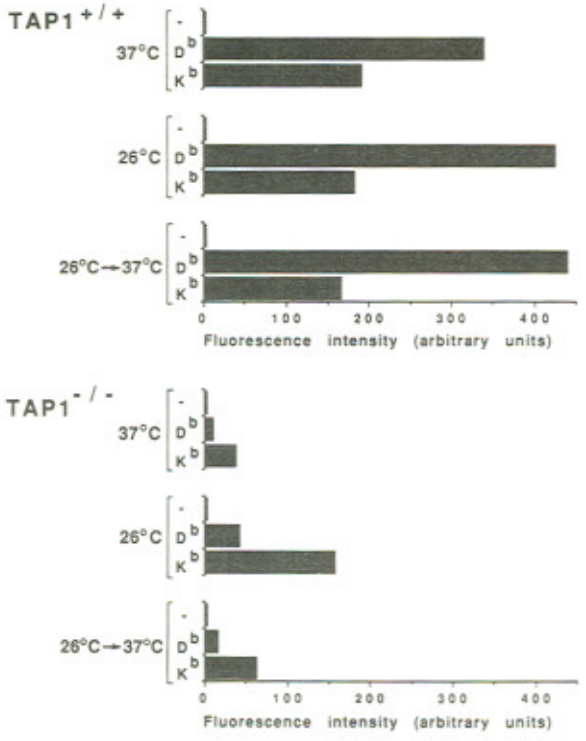

Figure 7. In Vitro Rescue of Class I Expression on *TAP1* Mutant Cells by Low Temperature

Concanavalin A–induced lymphoblasts from *TAP1*$^{+/+}$ or *TAP1*$^{-/-}$ mice were cultured for 24 hr at 37°C or 26°C and stained with an anti-D^b (B22-249.R1, α_1-specific) or anti-K^b (B8-24-3, α_1-specific) antibody followed by goat anti-mouse FITC-labeled immunoglobulin G and analyzed by FACS. As a control, cells were stained with the secondary antibody only (minus). To demonstrate that class I molecules induced at low temperature on the surface of *TAP1*$^{-/-}$ cells were devoid of peptide, cells cultured at 26°C were shifted to 37°C for 1 hr prior to staining.

analyses. A reduction of at least 30- to 40-fold in $CD4^-8^+$ T cells was observed (Figure 9), whereas the total number of $CD4^+8^-$ T cells seemed to be unaltered. Also, $CD4^+8^+$ thymocytes were present in normal numbers in mutant animals.

$CD4^-8^+$ T cells in mice have cytotoxic effector functions. We tested whether peripheral T cells from mutant mice could still be stimulated to produce alloreactive CTLs in bulk mixed lymphocyte cultures. Spleen cells from mutant mice were stimulated for 5 days with allogeneic (BALB/c and H-2^d) cells, and the presence of alloreactive CTLs was assessed in ^{51}Cr-release assays (see Figure 3C). CTL precursors were not detectable in spleen cells from mutant mice.

Discussion

Mutant cell lines with defects in the presentation of endogenous antigens to class I molecules have been valuable tools to study the mechanism of antigen processing and the rules that govern class I molecule assembly (Townsend et al., 1989b; Ljunggren et al., 1990a; Schumacher et al., 1990; Elliott, 1991). The defect in these cell lines could be explained by mutations in the postulated peptide transporter, TAP1–TAP2. In this study, we describe the phenotype of a mouse with a null mutation in the *TAP1* gene. Our data show that TAP1 is required for the cell surface expression, assembly, and intracellular transport of class I molecules. Several lines of evidence indicate that this phenotype is caused by a defect in the peptide loading of class I molecules. First, culture of *TAP1* mutant cells with synthetic peptides induces allele-specific class I expression on the cell surface as has been observed for the TAP2-defective RMA-S cells. Second, in detergent lysates from mutant cells, class I molecules are unstable at 37°C, and stability can be restored in an allele-specific manner by the addition of synthetic peptides, prior to exposure to 37°C. Third, a decrease in the temperature of culture (26°C) induces the transport of class I molecules to the cell surface. Finally, TAP1-deficient cells are unable to present endogenous antigens, as exemplified by cytoplasmic loading with OVA, to class I–restricted CTLs. We conclude therefore that the class I molecules in *TAP1* mutant cells are largely devoid of peptide.

The phenotype of the *TAP1* mutant cells closely resembles that of the mouse *TAP2* mutant cell line RMA-S and that of the human *TAP1* (721.134) and *TAP1–TAP2* (721.174 and T2) mutants. Our data, and studies with these cell lines, are consistent with a defect in intracellular peptide loading, transport, or both. The severe reduction in class I surface expression in TAP1-deficient mice demonstrates that TAP2 alone is not sufficient to allow peptide loading of class I molecules.

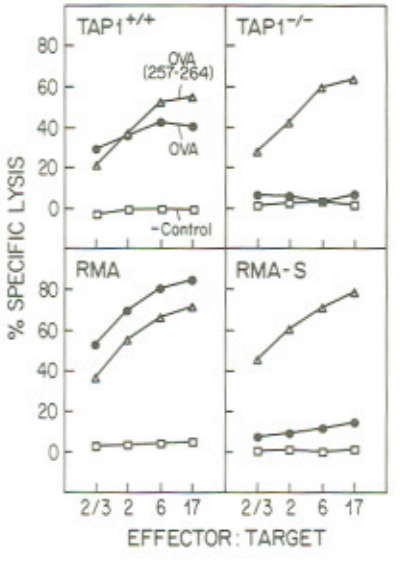

Figure 8. *TAP1*$^{-/-}$ Cells Are Deficient in Antigen Presentation

Lymphoblasts from *TAP1*$^{+/+}$ and *TAP1*$^{-/-}$ mice and from RMA and RMA-S cells were loaded with OVA (10 mg/ml) by osmotic lysis of pinosomes (closed circles) and tested for lysis by the OVA-specific CTL clone 4G3 in a ^{51}Cr-release assay. Controls were unloaded target cells (open squares) and targets treated with the OVA(257–264) peptide (1 μM) (open triangles).

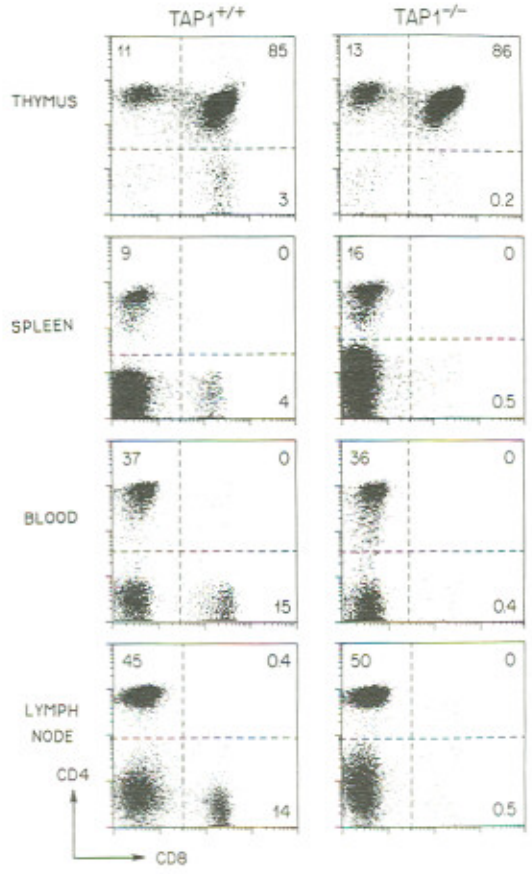

Figure 9. *TAP1*$^{-/-}$ Mice Lack CD4$^+$8$^+$ T Cells

Thymus, spleen, blood, and lymph node cells from mutant mice (−/−) or wild-type littermates (+/+) were stained for CD4- and CD8-expressing T cells. Cells were gated for lymphocytes. The numbers indicate the percentage of cells in each quadrant.

At least two alternative pathways for peptides to enter the ER can be envisaged. First, in selected cases, peptides derived from signal sequences of secreted or membrane-associated proteins could bind class I molecules, as shown for the human HLA-A2 molecules on T2 mutant cells (Henderson et al., 1992; Wei and Cresswell, 1992). The presentation of signal sequence peptides by HLA-A2 supposedly accounts for the relatively high levels (30% of wild type) of HLA-A2 observed on T2 and 721.174 cells. T2 cells transfected with *H-2 K*^{b} or *H-2 D*^{b} cDNA had no detectable peptides associated with K^b or D^b molecules (Wei and Cresswell, 1992). This observation, in addition to the very low levels of surface expression of K^b and D^b on both *TAP1*$^{-/-}$ and RMA-S cells, argues against an important role for this pathway in the expression of K^b and D^b molecules. However, an immunodominant T cell epitope from the lymphocytic choriomeningitis virus, presented by D^b, is derived from the signal sequence of a viral glycoprotein precursor (Buchmeier and Zinkernagel, 1992). Thus, we cannot completely rule out the involvement of signal sequence–derived peptides in the expression of mouse K^b and D^b molecules. Second, peptides could arise from cleavage by proteases in the lumen of the ER. An ER-specific degradation mechanism has been proposed (Lippincott-Schwartz et al., 1988), but, again, the absence of class I expression on *TAP1*$^{-/-}$ cells suggests that in the mouse only very few, if any, peptides entering the ER by this pathway are presented by class I molecules.

During their intrathymic differentiation, T cells undergo positive and negative cellular selection (Bevan, 1977; von Boehmer et al., 1978; Kappler et al., 1987). Positive selection ensures that mature T cells recognize antigen in the context of self-MHC molecules, and negative selection contributes to the establishment of self-tolerance. CD4$^-$8$^+$ cells were virtually absent in the thymus and peripheral lymphoid organs of mutant mice, and, as expected for mice without mature CD8$^+$ T cells, spleen cells from these mice could not induce a CTL response against alloantigens. This is consistent with studies on T cell receptor transgenic mice (Kisielow et al., 1988; Sha et al., 1988) and mice with a targeted mutation in the β_2-microglobulin gene (Koller et al., 1990; Zijlstra et al., 1990). Our data underscore the role of class I molecules in the positive selection of CD8$^+$ T cells.

Experiments with radiation chimeras (Nikolić-Žugić and Bevan, 1990) have suggested that self-peptides determine the selection of the T cell repertoire. Positive selection is thought to take place by interaction of the T cell receptor with MHC molecules expressed on epithelial cells of the thymic cortex (Fink and Bevan, 1978; Zinkernagel et al., 1978). Our results demonstrate that provision of peptides by TAP1–TAP2 is the predominant route by which class I molecules on thymus epithelial cells are loaded. We now have a system where the expression of class I molecules can be controlled by treatment with synthetic peptides. This should allow us to address directly the involvement of self-peptides in the repertoire selection, both in thymus organ cultures and in the intact animal.

Experimental Procedures

Cells

D3 ES cells (a gift from Dr. R. Kemler) from 129/Sv mice were maintained in the undifferentiated state by growth on a feeder layer of mitomycin C–treated embryonic fibroblasts carrying a *neo* resistance gene (Gossler et al., 1986) and culture medium supplemented with leukemia inhibitory factor (Smith et al., 1988). The RMA and RMA-S cell lines (obtained from Dr. K. Kärre) were derived from the Rauscher virus–transformed mouse lymphoma cell line RBL-5 (Ljunggren and Kärre, 1985; Kärre et al., 1986). The OVA-specific CTL clone 4G3 (Walden and Eisen, 1990) was established by Dr. C. Nagler-Anderson and was obtained from Drs. N. Fukusen and H. N. Eisen. This clone was maintained by weekly restimulation with irradiated E.G7-OVA cells (obtained from Dr. M. J. Bevan via Dr. H. N. Eisen).

Production of TAP1-Defective Mice

TAP1 was cloned from a C57BL/6 cosmid library (Stratagene) with a probe generated by the polymerase chain reaction (PCR). Two cosmids were isolated and mapped by restriction analysis. The targeting construct was prepared in a quatrimolecular ligation reaction, using a 6.5 kb EcoRI–SacII (the EcoRI site is within the cosmid vector) fragment from the 5′ end of *TAP1*, a 1.8 kb SacII–ClaI fragment containing a *neo* gene driven by the *PGK1* promoter (a gift from Dr. M. Rudnicki), a 2.5 kb BstXI–EcoRI fragment from the 3′ end of *TAP1*, and the plasmid pBluescript (Stratagene), digested with appropriate restriction enzymes. This construct is designed to delete a 7 kb fragment from the *TAP1* gene, encompassing nearly 80% of the protein-encoding region.

D3 ES cells were transfected with 50 μg of linearized targeting vector by electroporation (Bio-Rad Gene Pulser set at 800 V and 3 μF). G418 selection (150–175 μg/ml) was applied 24 hr after transfection, and G418-resistant colonies were isolated during days 7–10 of selection. Genomic DNA from these clones was digested with ScaI and hybridized with an 800 bp SmaI fragment from the 3′ *TAP1*-flanking region (Figure 1). Positive clones were confirmed by digestion with EcoRI and hybridization with a 550 bp HpaI–SphI fragment from the 5′ *TAP1*-flanking region (Figure 1).

Chimeric mice were generated as described by Bradley (1987). The contribution of ES cells to the germline of chimeric mice was determined by breeding with C57BL/6 mice and screening for agouti offspring. Germline transmission was confirmed by Southern blotting of tail DNA, and mice heterozygous for the mutation were interbred to homozygosity. Litters from heterozygous intercrosses were housed in autoclaved cages with autoclaved food and water.

Initial screening of mice was done by Southern blotting of tail DNA; later mice were typed by PCR analysis with a set of *neo* primers (5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAG-ATC-3′, 280 bp PCR fragment) and a set of primers to the deleted region of the mutant *TAP1* allele (5′-AGGCTCAGCGTGCCACTAAT-3′ and 5′-ATTGAAGTTCCTGCGCCTCC-3′, 240 bp PCR fragment).

Flow Cytometric Analyses

The following class I monoclonal antibodies were used: AF6-88.5 (D^b) (Pharmingen), H141-31-10 (D^b and α_2 specific) (Accurate), B22-249.R1 (K^b and α_1) (American Type Culture Collection [ATCC]), B8-24-3 (K^b and α_1) (ATCC), Y-3 (K^b and $\alpha_1+\alpha_2$) (obtained from Dr. H. N. Eisen), 20-8-4S (K^b and D^b) (ATCC), and 28-8-6S (K^b, D^b, α_1, and/or α_2) (ATCC). Other monoclonal antibodies were anti-CD4 (biotin labeled) and anti-CD8 (fluorescein isothiocyanate [FITC] labeled) (Pharmingen).

Cell suspensions from various tissues were prepared according to standard procedures. Cells (10^6) were incubated in staining buffer (phosphate-buffered saline, 1% bovine serum albumin, and 0.1% NaN_3) with monoclonal antibodies for 30 min at 4°C. Cells were then washed, and where appropriate, incubated with FITC-labeled goat anti-mouse immunoglobulin G (Southern Biotechnologies Associates) or streptavidin–PE (Pharmingen). After washing, 10,000 viable cells were analyzed using a FACScan flow cytometer (Becton-Dickinson). Dead cells were excluded from the analysis by staining with propidium iodide or based on the forward and sideways light scattering properties.

Immunoprecipitation Experiments

Cells were metabolically labeled with 500 μCi [^{35}S]methionine (1200 Ci/mol, Amersham) per 2×10^7 cells. For the pulse–chase experiment in Figure 4, cells were first starved for 1 hr in methionine-free medium and then labeled for 10 min at 37°C. After addition of 1 mM cold L-methionine, cells were further incubated at 37°C or 26°C. For each time point, 2×10^6 cells were harvested and lysed in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, and 30 TIU/ml aprotinin). For the experiment in Figure 5, cells were labeled for 4 hr at 37°C and lysed in Nonidet P-40 lysis buffer containing 10 mg/ml bovine serum albumin. Immunoprecipitation was performed as described (Ljunggren et al., 1990b). Antisera used are a rabbit serum raised against purified $H\text{-}2^b$ molecules (a gift from Dr. S. Nathenson) and a serum raised against a peptide from the cytoplasmic domain of H-2 K^b (Neefjes et al., 1992). Immunoprecipitates were analyzed by one-dimensional isoelectrofocusing as described (Ljunggren et al., 1990b).

Peptides

Amino acid sequences of synthetic peptides are as follows: VSV-NP peptide (amino acids 52–59), RGYVYQGL; SV-NP peptide (amino acids 324–332), FAPGNYPAL; IF-NP peptide (amino acids 366–374), ASNENMETM; OVA peptide (amino acids 257–264), SIINFEKL. Peptides were synthesized and high pressure liquid chromatography purified by the Biopolymers Laboratory (Howard Hughes Medical Institute, Massachusetts Institute of Technology).

Cytotoxicity Assays

Alloreactive CTLs were generated in 5 day bulk mixed lymphocyte cultures. Stimulators (5×10^6/ml) were irradiated (3000 rad) spleen cells, and responders (2×10^6/ml) were splenic cells. Targets were cell lines or splenic lymphoblasts induced with concanavalin A (2 μg/ml) for 2 days. For the experiment in Figure 8, 10 mg/ml OVA (grade VI; Sigma) was introduced in the cytoplasm of 10^6 target cells by the method of osmotic lysis of pinosomes (Moore et al., 1988) 3 hr before the ^{51}Cr-release assay.

The ^{51}Cr-release assay was performed by incubating 1×10^4 labeled target cells with serial dilutions of responders at 37°C for 4 hr. For the experiment in Figure 8, synthetic OVA(257–264) peptide (1 μM) was added to positive control cultures. Aliquots from the supernatant were counted in a γ counter. Results are presented as percentage of specific lysis: 100 × (experimental release − spontaneous release)/(release by detergent − spontaneous release). Spontaneous release was <25% of detergent release in all experiments. Each effector to target cell ratio was tested in duplicate.

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