

# Downregulation of Peptide Transporter Genes in Cell Lines Transformed with the Highly Oncogenic Adenovirus 12

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## Summary

The expression of class I major histocompatibility complex antigens on the surface of cells transformed by adenovirus 12 (Ad12) is generally very low, and correlates with the high oncogenicity of this virus. In primary embryonal fibroblasts from transgenic mice that express both endogenous H-2 genes and a miniature swine class I gene (PD1), Ad12-mediated transformation results in suppression of cell surface expression of all class I antigens. Although class I mRNA levels of PD1 and H-2D<sup>b</sup> are similar to those in nonvirally transformed cells, recognition of newly synthesized class I molecules by a panel of monoclonal antibodies is impaired, presumably as a result of inefficient assembly and transport of the class I molecules. Class I expression can be partially induced by culturing cells at 26°C, or by coculture of cells with class I binding peptides at 37°C. Analysis of steady state mRNA levels of the TAP1 and TAP2 transporter genes for Ad12-transformed cell lines revealed that they both are significantly reduced, TAP2 by about 100-fold and TAP1 by 5–10-fold. Reconstitution of PD1 and H-2D<sup>b</sup>, but not H-2K<sup>b</sup>, expression is achieved in an Ad12-transformed cell line by stable transfection with a TAP2, but not a TAP1, expression construct. From these data it may be concluded that suppressed expression of peptide transporter genes, especially TAP2, in Ad12-transformed cells inhibits cell surface expression of class I molecules. The failure to fully reconstitute H-2D<sup>b</sup> and H-2K<sup>b</sup> expression indicates that additional factors are involved in controlling class I gene expression in Ad12-transformed cells. Nevertheless, these results suggest that suppression of peptide transporter genes might be an important mechanism whereby virus-transformed cells escape immune recognition in vivo.

MHC class I genes play key roles in numerous immunological processes. Among these are the presentation of “foreign” antigens for recognition by CTLs (1). By this mechanism, the immune system is able to control infectious diseases and the growth of tumor cells (2, 3). Indeed, tumors of various origin have been shown to express low levels of class I antigens, a characteristic that might contribute to their escape from immune surveillance (4). In support of this, Tanaka et al. (5) reported that the reexpression of class I antigens inhibited tumor growth. However, transfection of cell lines by class I genes does not always result in the expression of class I molecules on the cell surface. Weis and Seidman (6) reported that transfection of L cells by a class I gene resulted in increased levels of class I mRNA, but failed to significantly increase the cell surface expression of class I antigens. Thus, the suppressed expression of class I molecules by cells may

occur during transcription of the genes, or during synthesis, assembly, and transport to the cell surface.

Transport of class I molecules to the cell surface depends on their assembly with peptides, which are usually 8–9 amino acids long (7). Several researchers (8–10) have demonstrated that such peptides arise from cleavage of proteins in the cytosol and are actively transported into the endoplasmic reticulum (ER)<sup>1</sup>, where assembly with class I molecules takes place. Presumably, if the antigen processing machinery of the cell is functioning normally, the tumor-associated antigens and antigens originating from oncogenic viruses generate such peptides (11–13). After assembly, the molecules are transported through the Golgi apparatus to the cell surface. Klar and Hämmerling (14) showed that tumor cells, such as the lung carcinoma CMT 64.5 and the fibrosarcoma BC2, can synthesize

R. Rotem-Yehudar and S. Winograd made equal contributions to this paper.

<sup>1</sup> Abbreviations used in this paper: Ad12, adenovirus 12;  $\beta_2m$ ,  $\beta_2$ -microglobulin; ER, endoplasmic reticulum; F.U., fluorescence units; MEF, mouse embryonal fibroblasts.

class I heavy chains and  $\beta_2$ microglobulin ( $\beta_2m$ ), but that these are only assembled after treatment with  $\gamma$ -interferon. Furthermore, mutant cells with deletions or mutations in the MHC locus, but with intact class I genes, such as the murine RMA-S and the human T2 or 721.174 cells, have drastically reduced cell surface levels of class I, as a result of a defect in peptide transport into the ER (10, 15, 16). Expression can be restored by transfecting the cells with the MHC-encoded peptide transporters TAP1 and TAP2 (17, 18). Recently, Restifo et al. (19) demonstrated that tumor cell lines differ in their ability to process antigens, a phenomenon that is correlated with poor class I expression and low mRNA levels of both peptide transporters and proteasome components. However, this study did not address the question of whether poor antigen processing is associated with transformation, or represents the natural regulation of class I expression in the tissue of origin.

Tumors transformed by the highly oncogenic adenovirus 12 (Ad12) have been used as model systems to demonstrate a direct correlation between tumorigenicity and lack of host immune responses due to decreased class I expression (20–22). Whereas both Ad5 and Ad12 transform primary fibroblasts in vitro, tumors are generated in vivo only by Ad12-transformed cells that are deficient in class I expression. Downregulation of class I expression was reported to occur at both the transcriptional (23–26) and posttranscriptional levels (27). In our previous reports we described a large panel of cell lines transformed by Ad12, and compared them to both Ad5-transformed cell lines and immortalized (“normal”) cell lines derived from the same pool of fibroblasts (27). The parental fibroblasts were prepared from transgenic mice which express both endogenous class I genes and a miniature swine class I transgene (PD1) that is not located in the MHC locus. Although all cell lines express class I mRNA, the levels of class I antigens, but not other receptors (such as MAC-2 and CD44), are significantly decreased in most Ad12-transformed cell lines. Comparison of the three class I gene transcripts in Ad12-transformed cells revealed that the levels of PD1 and H-2D are about normal, whereas the H-2K level is two- to fivefold lower (Winogard, S., R. Rotem-Yehudar, and R. Ehrlich, manuscript in preparation). Immunoprecipitation analyses with or without endo- $\beta$ -N acetylglucosaminidase H (endo H) treatment, revealed that Ad12-transformed cell lines are deficient in both class I molecule synthesis and the ability to transport these molecules through the Golgi apparatus (28). Both H-2 and PD1 transport were inhibited, implying the existence of a general mechanism that affects maturation of all class I products in these cells. These results raised the possibility that transformation by certain viral serotypes can affect peptide transport. In this paper, we pursue the causes of aberrant transport and expression of class I molecules in Ad12-transformed cells.

## Materials and Methods

**Mice.** C57Bl/10 mice, which are transgenic for a miniature swine class I antigen, PD1 (PD1 transgenic mice), have been previously described (27, 29). The mice, which are homozygous for the transgene, were bred at the Tel Aviv University breeding facility.

**Cell Cultures.** All the cell lines were derived from cultured mouse embryonal fibroblasts (MEF) as previously described (27). M1 was derived from spontaneous in vitro immortalization of MEF after growth crisis (27). VAD12.78, VAD12.79, and VAD12.42 were transformed by infection of MEF with Ad12 (27). A5O1, A5O3, and A5O5 were transformed by transfection of MEF with Ad5-Xhol-C fragment in a PSV2Neo plasmid (30, 31), followed by selection with 800  $\mu$ g/ml of G418 (Sigma Chemical Co., St. Louis, MO). The plasmids were a gift from Dr. A. Van der Eb (University of Leiden, Leiden, The Netherlands). ME1 and ME5 were derived from transformation of BALB/c-MEF by Ad5 and were a gift from Dr. A. M. Lewis (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD) (32).

The cell lines were maintained in DMEM, supplemented with 2 mM glutamine, 10% FCS, penicillin, streptomycin, gentamycin, and amphotericin B at the recommended concentrations (33). Media and supplements were purchased from Biological Industries, Bet Haemek, Israel.

**Stable Transfection.** Ad12-transformed cells (VAD12.79) were transfected by the calcium phosphate-DNA coprecipitation method (28). The transfection cocktail contained 10  $\mu$ g plasmid DNA and 5  $\mu$ g of carrier DNA (sheared salmon sperm DNA; Sigma Chemical Co.). 24 h after transfection, the cells were washed with PBS, and after a further 24 h the medium was supplemented with 800 mg/ml G418 (Sigma Chemical Co.). The transfected cells were either grown to confluence and analyzed as a bulk mixture of cells, or individual clones were isolated and expanded in culture.

**Probes and Plasmids.** The PD1-specific probe is a SacI–BamHI fragment containing exon 2–7 of the PD1 gene (29); the H-2 probe is an EcoRI–HindIII fragment derived from pH-2<sup>d</sup>33 (H-2K<sup>d</sup>) (34); the  $\beta_2m$  probe is a PstI–PstI fragment from  $\beta_2m$  cDNA cloned in pBR322 (35); the actin probe is a PstI–PstI fragment from chicken  $\beta$  actin cDNA cloned in pBR322 (36); the Ad5E1a probe is an EcoRI–EcoRI fragment containing the E1a of Ad5 cloned in RSVNeo and was a gift from Dr. A. Van der Eb (University of Leiden); and the TAP1 and TAP2 probes are XbaI–HindIII and KpnI–KpnI fragments containing TAP1 and TAP2 cDNAs, respectively cloned in pcDNA1 Neo (Invitrogen, San Diego, CA), were a gift from Dr. J. J. Monaco (University of Cincinnati, Cincinnati, OH) (37).

**Antibodies.** The antibodies used for FACS<sup>®</sup> analyses and their specificities are: 20.8.4S, which recognizes H-2K<sup>b</sup> and H-2K<sup>d</sup>; 28.14.8, which recognizes an epitope on  $\alpha 3$  domain of H-2D<sup>b</sup> and H-2L<sup>d</sup> heavy chains with or without associated  $\beta_2m$  (38–40); 27.11.13, which recognizes H-2D<sup>b</sup> and H-2D<sup>d</sup>; B8.24.3, which recognizes H-2K<sup>b</sup>; and PT85A, which recognizes a public determinant on swine lymphocyte antigens (SLA) (41). FITC-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Induction of Class I Expression.** The cells were incubated for 18 h with the peptides listed in Table 1 in serum-free media (Bio MPM 1, multipurpose serum-free medium for adherent cells; Biological Industries). Peptides were synthesized, purified, and analyzed as previously described (42).

**FACS<sup>®</sup> Analysis.** The cells were harvested by mild trypsinization, followed by washing with media supplemented with 5% FCS and 0.01% sodium azide. Approximately  $10^6$  cells were incubated at 4°C with the appropriate concentration of the first antibody for 30 min, washed, and then incubated in the dark for another 30 min with the second antibody. Controls were stained with a first nonrelevant antibody and a second antibody. The cells were washed with PBS and the fluorescence intensity analyzed by a cell sorter (Becton Dickinson & Co., Mountain View, CA).

**Table 1.** Peptides and Their MHC Restriction

Peptide	Position*	Restriction	Sequence	Reference
Flu NP	366-374	D <sup>b</sup>	ASNENMETM	43-45
Ad5 E1a	234-243	D <sup>b</sup>	SGPSNTPPE I	11, 45
VSV NP	52-59	K <sup>b</sup>	RGVYVYQGL	7, 45
OVA	257-264	K <sup>b</sup> (D <sup>b</sup> ) <sup>†</sup>	S I I NFEKL	45, 46
Sendai NP	324-332	K <sup>b</sup> (D <sup>b</sup> ) <sup>†</sup>	FAPGNYPAL	45, 47
Flu PR8 NP	147-155	K <sup>d</sup>	TYQRTRALV	44, 45
Flu JAP HA	529-537	K <sup>d</sup>	I YATVAGSL	45, 48, 49
Flu PR8 HA	533-541	K <sup>d</sup>	I YSTVASSL	45, 50
p18-HIV	318-327	D <sup>d</sup>	RGPGRAFVT I	51
Turn-p35B	133-141	D <sup>d</sup>	NGPPHSNNF	52
CMV	168-176	L <sup>d</sup>	YPHFMPNTL	53

\* Residue number of the peptide in the native protein.

<sup>†</sup> Sendai NP 324-332 is restricted to H-2K<sup>b</sup> but it also was shown to stabilize H-2D<sup>b</sup> cell surface expression (54, 55). OVA 257-264 was shown to promote the expression of H-2K<sup>b</sup> and to some extent H-2D<sup>b</sup> (55).

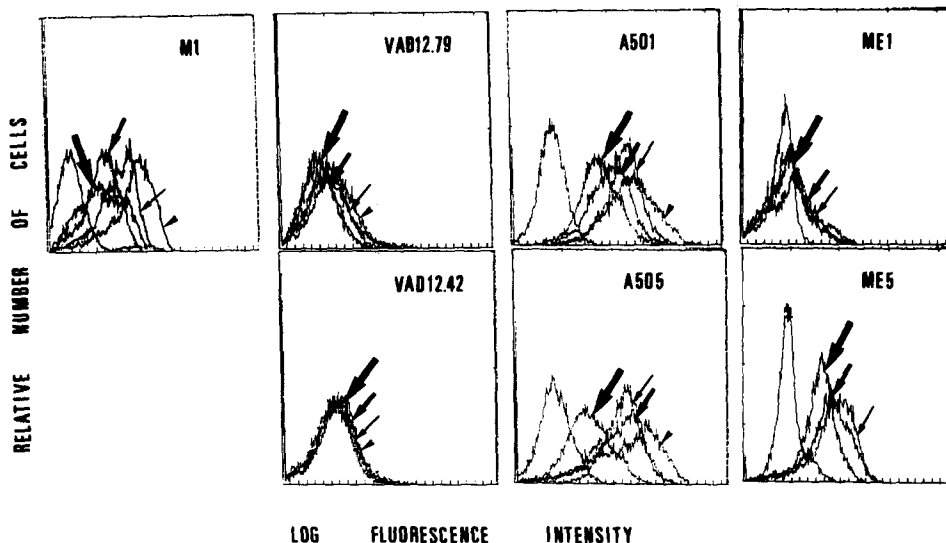
**RNA Analysis.** Cytoplasmic RNA was prepared by the method of White and Bancroft (56) as previously described (27). Enrichment for poly A mRNA was done according to Sambrook et al. (57). 10  $\mu$ g of poly A-enriched mRNA was denatured and run on a 1.2% formaldehyde/formamide agarose gel, blotted into Hybond-N membrane (Amersham International, Little Chalfont, UK) and hybridized with the appropriate probe, which was labeled with  $\alpha$ -[<sup>32</sup>P]dCTP (Rotem Industries, Diom, Israel) using a Random Priming labeling kit (United States Biochemical Corp., Cleveland, OH).

The hybridization solution contained 4  $\times$  SSC, 50% formamide, 0.2% SDS, 0.1% polyvinylpyrrolidone, and 100  $\mu$ g/ml sheared salmon sperm DNA. Hybridizations were carried out at 42°C followed by washes with 2  $\times$  SSC, 0.1% SDS at room temperature, and 0.2  $\times$  SSC in temperatures ranging between 55 and 65°C. The blot was exposed to RX x-ray films (Fuji, Tokyo, Japan) and the resulting bands were scanned by a densitometer.

The blot, after stripping with a boiling solution of 0.1% SDS, was used seven times for hybridizations.

## Results

**Cell Surface Expression of Class I Antigens Is Decreased After Transformation with Ad12.** The following studies were carried out with a representative normal cell line obtained by spontaneous immortalization of MEF from C57Bl/10.PD1 mice (M1), Ad12- and Ad5E1-transformed MEF from the same mice (27, 28), and Ad5-transformed MEF from BALB/c mice (32). The cell surface level of expression and the percent positive cells for H-2 antigens and the transgene product PD1 were compared in the adenovirus-transformed and the normal cell lines (Fig. 1 and Table 2). The Ad12-transformed cell lines VAD12.42 and VAD12.79 expressed very low levels of all class I antigens, as reflected both by the percent of positive cells in the population (Table 2) and the relative fluorescence per cell (Fig. 1 and Table 2). Cell lines derived by transformation of MEF with Ad5E1 (A5O1 and A5O5) expressed higher levels of class I antigens than M1. For comparison, we ana-



**Figure 1.** Cell surface expression of class I antigens is decreased after transformation with Ad12. Cell surface expression of class I antigens on the normal cell line (M1), Ad12-transformed cell lines (VAD12.42, VAD12.79), Ad5E1-transformed cell lines (A5O5, A5O1), and Ad5-transformed cell lines (ME1, ME5) was analyzed by immunofluorescence staining with the following antibodies: PT85A (anti-PD1) ( $\blacktriangledown$ ), 20.8.4S (anti-H-2K<sup>b</sup>, anti-H-2K<sup>d</sup>) ( $\longleftrightarrow$ ), 28.14.8 (anti-H-2D<sup>b</sup>, anti-H-2L<sup>d</sup>) ( $\rightarrow$ ) and 27.11.13 (anti-H-2D<sup>b</sup>, anti-H-2D<sup>d</sup>) ( $\longleftrightarrow$ ). The negative control (staining with a nonrelevant first antibody) is not marked with an arrow.

**Table 2.** Cell Surface Expression of Class I Antigens Is Decreased after Transformation with Ad12

Cell line*	Percent positive cells (mean F.U./Cell)†			
	Antibody			
	PT85A (anti-PD1)	20.8.4S (anti-H-2K <sup>b</sup> ,K <sup>d</sup> )	27.11.13 (anti-H-2D <sup>b</sup> ,D <sup>d</sup> )	28.14.8 (anti-H-2D <sup>b</sup> ,L <sup>d</sup> )
M1	84 (150)	54 (20)	69 (35)	80 (60)
VAD12.79	22 (15)	3 (9)	20 (15)	32 (15)
VAD12.42	0 (4)	0 (4)	0 (4)	0 (4)
A5O1	95 (900)	85 (150)	91 (200)	97 (600)
A5O5	85 (600)	62 (60)	93 (200)	83 (200)
ME1	—	35 (15)	38 (15)	39 (15)
ME5	—	83 (150)	81 (150)	78 (150)

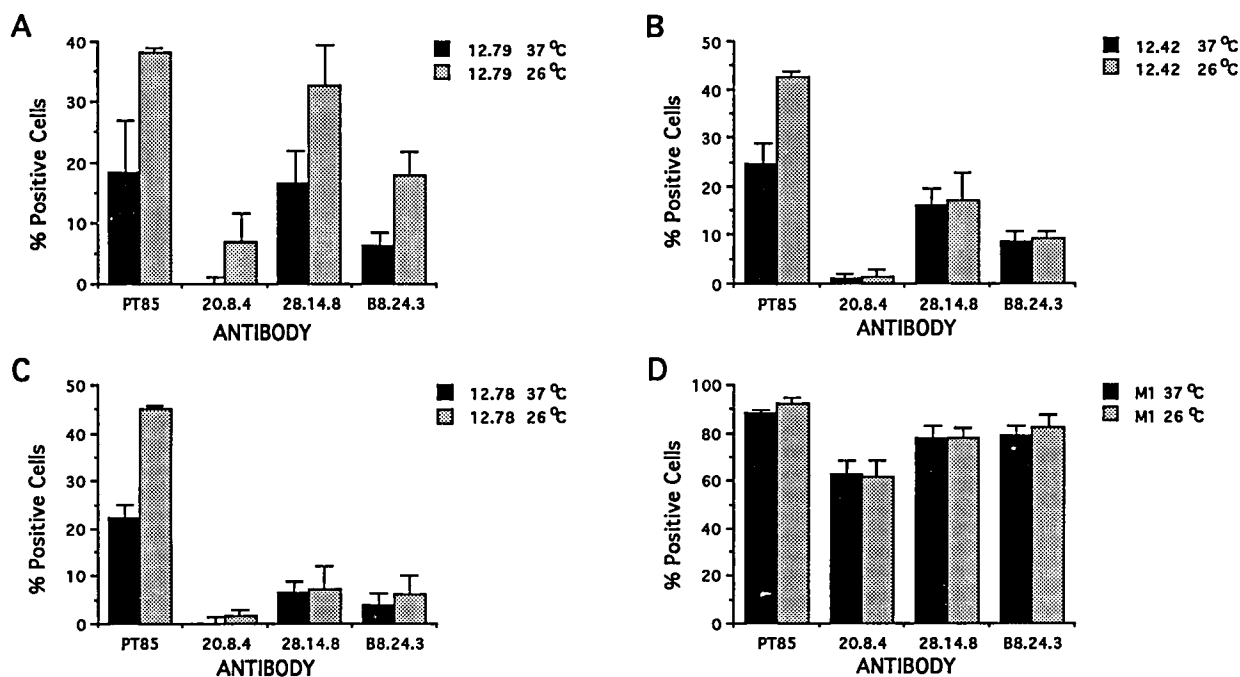
\* M1 is a normal cell line; VAD12.79 and VAD12.42 are Ad12-transformed cell lines; A5O1 and A5O5 are E1Ad5-transformed cell lines; ME1 and ME5 are Ad5-transformed cell lines.

† The percent of positive cells and their mean fluorescence were determined by comparing with the staining obtained with a nonrelevant first antibody.

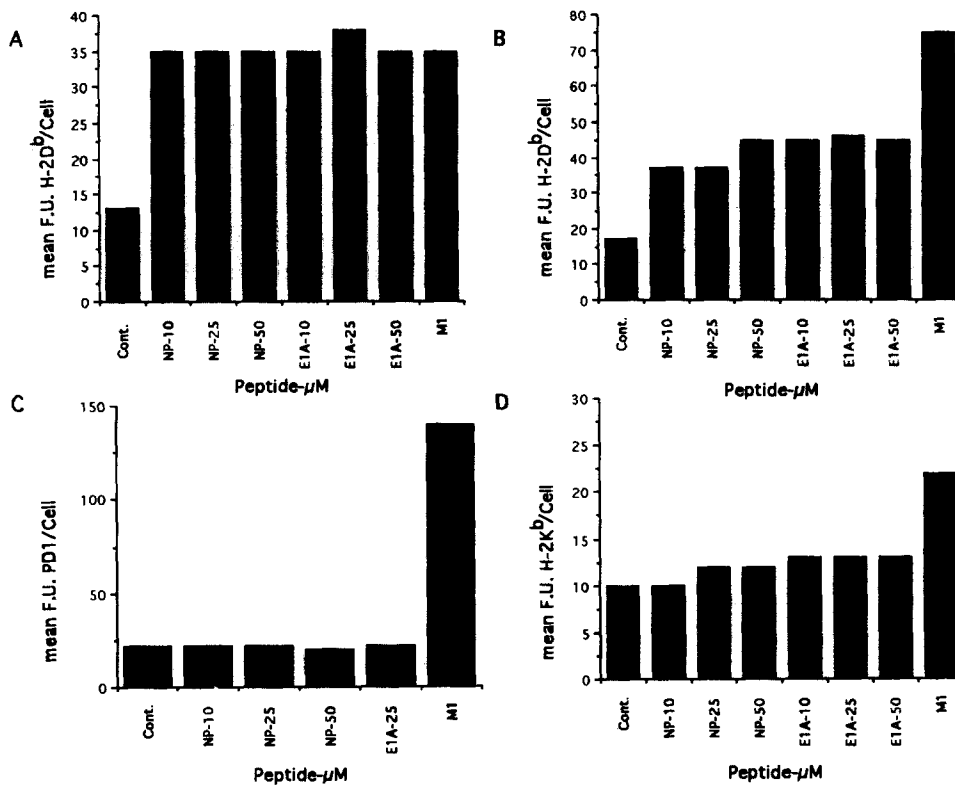
lyzed the Ad5-transformed cell lines ME1 and ME5 described by Haddada et al. (32) who also showed that ME5 expresses higher levels of class I antigens than ME1.

**Effects of Low Temperature on Expression of Class I Epitopes on the Surface of Ad12-transformed Cells.** Expression of class I molecules on the surface of RMA-S cells can be enhanced by culture at lower temperatures (58). To determine whether the level of class I molecules on the surface of Ad12-transformed cells is increased after incubation at reduced temperatures, we cultured the cell lines for 18 h at 26°C and examined cell surface expression by FACS® analyses. As shown in Fig. 2, class I expression was not enhanced in the normal cell line (M1), whereas the expression of epitopes recognized by PT85A (anti-PD1) were enhanced in Ad12-transformed cell lines. A two-fold increase in the mean fluorescence/cell of PD1-positive cells was also observed (data not shown). Nevertheless, PD1 expression reached <50% of the maximal level expressed in M1 cells. Only one cell line (VAD12.79) demonstrated an increased number of H-2 positive cells after incubation at 26°C (Fig. 2), but even for this cell line the mean fluorescence/cell did not change significantly (data not shown).

**Specific Peptides Induce Expression of Class I Molecules on the Surface of Ad12-transformed Cells.** The enhanced expression of PD1 at lower temperatures raised the possibility that peptide transport is inefficient in these cells. Class I molecules in cells lacking peptide transporters, and in turn, exhibiting low levels of the relevant peptides in the ER, may be stabilized by extracellular peptides (43, 47). To determine whether this was the case with our cell lines, we cultured the cells in the presence of peptides known to bind H-2D<sup>b</sup> or H-2K<sup>b</sup>. To prevent enzymatic degradation, or effects mediated by bovine β<sub>2</sub>m, all the incubations were done in serum-free media. The peptides used are listed in Table 1. Two H-2D<sup>b</sup>-



**Figure 2.** Effects of incubation at reduced temperature on cell surface expression of class I antigens in Ad12-transformed cell lines. Cell surface expression of PD1, H-2K<sup>b</sup>, and H-2D<sup>b</sup> in Ad12-transformed cell lines (VAD12.79, VAD12.42, VAD12.78) (A-C), and the immortalized cell line (M1) (D) was analyzed after incubation of cells at 37°C (■) and 26°C (▨). The antibodies used were PT85A (anti-PD1), 20.8.4S (anti-H-2K<sup>b</sup>), B8.24.3 (anti-H-2K<sup>b</sup>), and 28.14.8 (anti-H-2D<sup>b</sup>).



**Figure 3.** Effects of H-2D<sup>b</sup>-specific peptides on cell surface expression of class I antigens in Ad12-transformed cell lines. Cell surface expression of H-2D<sup>b</sup> (A and B), PD1 (C), and H-2K<sup>b</sup> (D) on the Ad12-transformed cell line VAD12.79 was analyzed after incubation of cells for 18 h with serum-free medium (Cont.), or with the Flu NP (NP) or Ad5 E1a (E1a) peptides at various concentrations (10, 25, and 50  $\mu$ M, as indicated in the figure) and compared with that of M1 incubated in serum-free medium (M1). The antibodies used were 27.11.13 (anti-H-2D<sup>b</sup>) (A), 28.14.8 (anti-H-2D<sup>b</sup>) (B), PT85A (anti-PD1) (C), and 20.8.4 (anti-H-2K<sup>b</sup>) (D).

and three H-2K<sup>b</sup>-restricted peptides were used; however, the H-2K<sup>b</sup>-restricted peptides, Sendai NP 324-332 and Ova 257-264, have been also shown to somewhat stabilize H-2D<sup>b</sup> expression (54, 55). Two H-2D<sup>d</sup>, three H-2K<sup>d</sup>, and one H-2L<sup>d</sup>-specific peptides were used to analyze effects on class I expression by ME1 cells, which are of BALB/c origin.

Whereas Fig. 3, A and B show that the H-2D<sup>b</sup>-specific peptides enhanced H-2D<sup>b</sup> expression on VAD12.79 by two- to threefold, resulting in percent positive cells equal to that in M1 (data not shown), PD1 and H-2K<sup>b</sup> expression were not further enhanced by these peptides (Fig. 3, C and D). Similar results were observed with the addition of H-2D<sup>b</sup>-specific

**Table 3.** H-2D<sup>b</sup>-restricted Peptides Induce Specific Class I Expression on Ad12-transformed Cells

Antibody	Peptide*	Cell Line			
		M1	A5O1	VAD12.79	VAD12.42
		Percent Positive Cells $\pm$ S.E.†			
PT85A (anti-PD1)	-	76.0	89.5 $\pm$ 5.5	39.3 $\pm$ 9.9	15.5 $\pm$ 0.5
	+	81.0	82.5 $\pm$ 6.5	38.7 $\pm$ 9.3	24.5 $\pm$ 7.5
20.8.4S (anti-H-2K <sup>b</sup> )	-	86.0	76.0 $\pm$ 2.0	7.0 $\pm$ 5.1	4.5 $\pm$ 4.5
	+	72.0	72.5 $\pm$ 3.5	5.7 $\pm$ 3.2	3.5 $\pm$ 3.5
27.11.13 (anti-H-2D <sup>b</sup> )	-	87.0	71.0 $\pm$ 4.0	21.0 $\pm$ 9.9	3.5 $\pm$ 3.5
	+	80.0	90.5 $\pm$ 1.5	47.3 $\pm$ 9.6	44.5 $\pm$ 11.5
28.14.8 (anti-H-2D <sup>b</sup> )	-	77.0	88.0 $\pm$ 2.0	29.6 $\pm$ 9.2	9.0 $\pm$ 0.05
	+	88.0	79.5 $\pm$ 5.5	49.3 $\pm$ 10.1	52.5 $\pm$ 2.5

\* Treatment with peptides (20  $\mu$ M) and FACS<sup>®</sup> analysis were done as described in Materials and Methods.

† S.E., Standard Error.

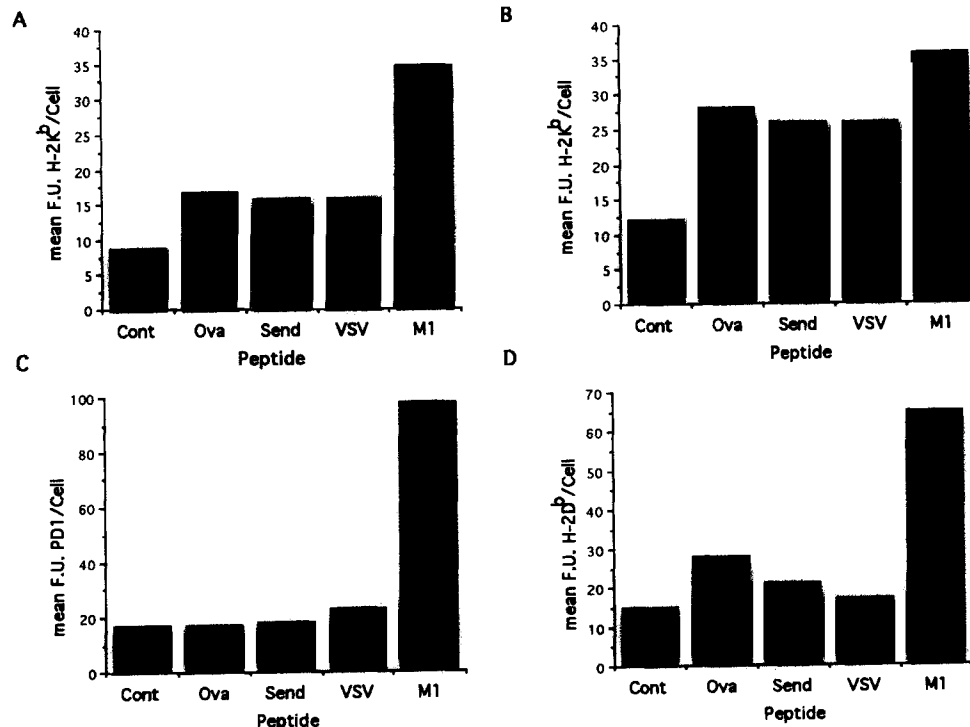
peptides to VAD12.42 cells (Table 3). None of the peptides induced additional class I expression on M1 or E1Ad5-transformed cell lines (Table 3).

A two- to threefold enhancement of class I expression could be obtained for H-2K<sup>b</sup>, but required the addition of higher concentrations of H-2K<sup>b</sup>-specific peptides (150 μM) (Fig. 4). In contrast to the results obtained following treatment by H-2D<sup>b</sup>-specific peptides where more than 50% of the cells expressed detectable levels of class I antigens, cells expressing detectable levels of H-2K<sup>b</sup> after treatment with the relevant peptides consisted only 15–35% of the population. The fact that H-2K<sup>b</sup> expression could not be induced to the same extent as the H-2D<sup>b</sup> expression is consistent with the observed transcriptional downregulation of the H-2K<sup>b</sup> gene (Winograd et al., manuscript in preparation). Treatment with OVA 257–264 resulted in some induction of H-2D<sup>b</sup> expression as expected, but only at the highest peptide concentration (150 μM). H-2K<sup>b</sup>-specific peptides did not affect PD1 expression. In contrast to the induction of cell surface expression of class I molecules after the treatment of Ad12-transformed cells with specific peptides, the levels of class I antigens on the surface of the Ad5-transformed cell lines, ME1 and ME5, the Ad5E1-transformed and M1 cell lines, were not increased by incubation with the peptides listed in Table 1 (data not shown). Thus, decreased cell surface expression of class I antigens in the Ad12-, but not in the Ad5-transformed cell lines is apparently caused by a lack of peptides in the ER.

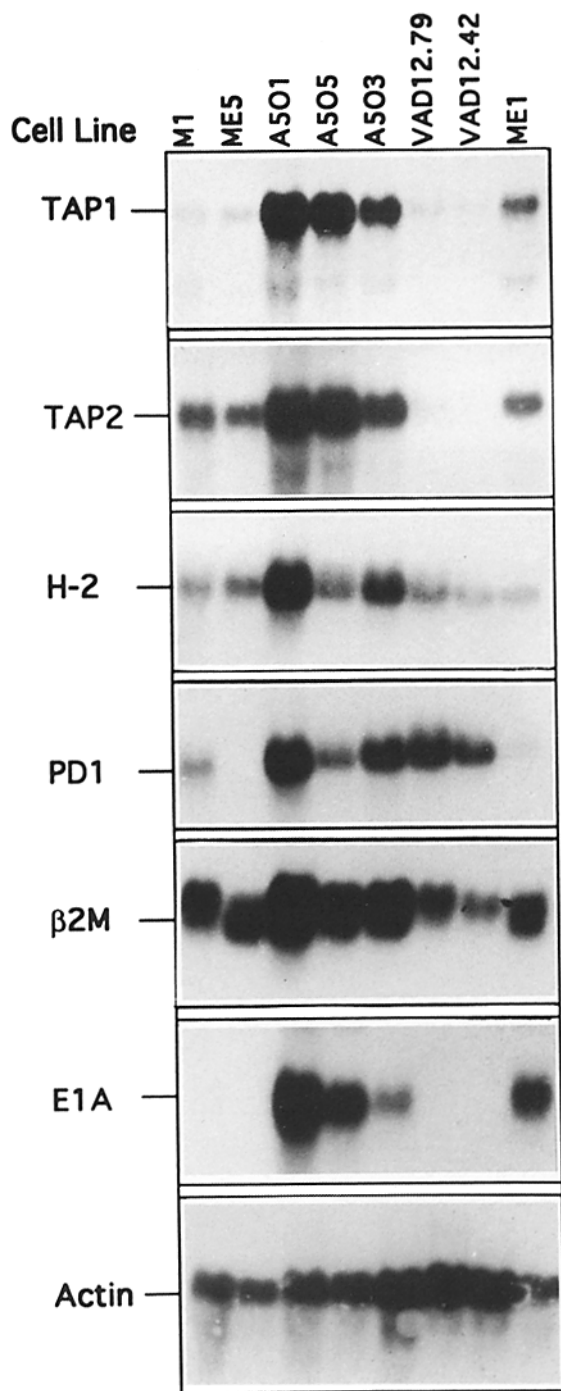
Preincubation of cells with exogenous β<sub>2</sub>m was previously shown to facilitate the association of peptides with class I heavy chains (58–60). To examine the effect of such treat-

ment on our cell lines they were incubated with recombinant human β<sub>2</sub>m, or with a mixture of peptides and human β<sub>2</sub>m. Neither the addition of human β<sub>2</sub>m alone, nor human β<sub>2</sub>m plus the highest tested peptide concentration, resulted in further enhancement of class I expression (results not shown). Thus, exogenously added β<sub>2</sub>m did not facilitate class I expression in this system.

**Suppression of Peptide Transporter Genes in Ad12-transformed Cells.** The apparent deficiency of peptides for assembly and transport of class I molecules to the cell surface, raised the possibility that peptide transporter genes are downregulated in Ad12-transformed cell lines. Hybridization of total RNA from seven individual Ad12-transformed cell lines with TAP derived probes revealed that the level of peptide transporter mRNA, particularly TAP2, is significantly decreased compared to that in Ad5-transformed cell lines. However, because the expression of both TAP1 and TAP2 was very low in MEF and MEF derived cell lines, poly A mRNA was isolated in order to quantitatively compare TAP expression in the different cell lines. Fig. 5 and Table 4 summarize the results of Northern hybridization analyses of poly A mRNA isolated from the various cell lines with class I, β<sub>2</sub>m, Ad5E1a, and peptide transporter-derived probes. The most marked difference between Ad12-transformed and the other cell lines was in the level of expression of the TAP2 gene. The hybridization signal with the TAP2 probe was very low in Ad12-transformed cells (at least a 100-fold decrease in TAP2 mRNA in VAD12.42 and VAD12.79 as compared with M1). In cell lines transformed by Ad5E1 (A5O1 and A5O5), there was a four- to fivefold increase in the steady state level of TAP2, compared



**Figure 4.** Effects of H-2K<sup>b</sup>-specific peptides on cell surface expression of class I antigens in Ad12-transformed cell lines. Cell surface expression of H-2K<sup>b</sup> (A and B), PD1 (C), and H-2D<sup>b</sup> (D) on the Ad12-transformed cell line VAD12.79 was analyzed after incubation of cells for 18 h in serum-free medium (Cont) or with 150 μM of OVA, Sendai NP (Send), and VSV NP (VSV) and compared with that of M1 incubated in serum-free medium (M1). The antibodies used were 20.8.4S (anti-H-2K<sup>b</sup>) (A), B8.24.3 (anti-H-2K<sup>b</sup>) (B), PT85A (anti-PD1) (C), and 28.14.8 (anti-H-2D<sup>b</sup>) (D).



**Figure 5.** Expression of peptide transporter genes is suppressed in cell lines transformed by Ad12. Electrophoresis on formaldehyde/formamide agarose gel was carried out with 10  $\mu$ g of poly A enriched mRNA from the normal cell line (M1), Ad12-transformed cell lines (VAD12.79, VAD12.42), Ad5E1-transformed cell lines (A5O1, A5O3, A5O5), and Ad5-transformed cell lines (ME1, ME5). RNA was transferred to nylon membrane and hybridized as indicated in Materials and Methods with probes specific for the following transcripts; TAP1, TAP2, H-2, PD1,  $\beta_2$ M ( $\beta_2$ M), Ad5E1a (E1A), and actin. The blot was stripped between hybridizations as indicated in Materials and Methods. The results of the densitometric analysis are summarized in Table 4.

with M1. ME1 and ME5, which originated from Ad5-transformation of BALB/c fibroblasts and one of the Ad5E1 transformed cell lines (A5O3), expressed similar levels of TAP2 mRNA to those in M1.

TAP1 gene expression was suppressed by 5–10-fold in Ad12-transformed, compared with M1 cells. By contrast, in two cell lines transformed by Ad5E1 (A5O1 and A5O5) there was a 10-fold increase in the steady state level of TAP1 transcripts in comparison to M1 cells. A two- to threefold induction in TAP1 expression also occurred in A5O3 and ME1. One of the major differences between ME1 and ME5 is the absence of E1a expression in ME5 (Fig. 5), although E1b mRNA is expressed (results not shown). The absence of E1a proteins in ME5 and the low level of E1a in A5O3 could relate to the difference in TAP1-gene expression between the cell lines.

*Reconstitution of Class I Expression in an Ad12-transformed Cell Line Stably Transfected with a TAP2 Expression Vector.* To determine whether class I expression can be reconstituted by expression of peptide transporter genes, an Ad12 transformed cell line (VAD12.79), was transfected with either TAP1, TAP2, a mixture of TAP1 and TAP2 expression constructs, or a PSV2Neo control construct. TAP1 and TAP2 were transcribed from the CMV promoter in these constructs. Since the neomycin gene and the TAP cDNAs were located on the same plasmid, all transfectants were pooled and class I expression was analyzed by FACS<sup>®</sup>. In addition, several individual clones were expanded in culture and analyzed. The FACS<sup>®</sup> analyses of the bulk transfected cultures is shown in Fig. 6, B–D. TAP1 transfection did not increase the expression of any of the class I molecules (Fig. 6 C compared with A and B) although the level of TAP1 transcripts was 10-fold higher in the transfected cells than in M1 (data not shown). TAP2 transfection partially reconstituted class I expression in the bulk culture cells (Fig. 6 D). PD1 expression was increased on 55% of the cells and mean fluorescence units (F.U.)/cell increased from 9 to 22 (Fig. 6 D1). The pattern of PD1 expression suggested the existence of three subpopulations in the pool of transfectants: a small subpopulation with negative PD1 expression, a large subpopulation with enhanced PD1 levels/cell, and a population of ~25% of the cells that demonstrated similar PD1 levels to those in M1 (compare with Fig. 6 F). The reconstitution of expression of H-2 antigens was less striking. TAP2 transfection increased H-2D<sup>b</sup> expression on 35% of the cells with only a slight increase in mean F.U./cell (Fig. 6, D2 and D3), but did not induce the expression of H-2K<sup>b</sup> (Fig. 6 D4). Transfection of the cell line with a mixture of TAP1 and TAP2 did not induce higher levels of class I expression than transfection with TAP2 alone (data not shown). Fig. 6 E shows the FACS<sup>®</sup> analyses of an individual clone transfected with TAP2. In this clone, PD1 expression was completely reconstituted (Fig. 6 E1), and H-2D<sup>b</sup> molecules were expressed in 85% of the cells (Fig. 6, E2 and E3). However, the mean F.U. of H-2D<sup>b</sup> molecules/cell did not reach the same as M1 (27 F.U./cell in the TAP2 transfected clone and 92 F.U./cell in M1). The expression of H-2K<sup>b</sup> molecules was not reconstituted (Fig. 6 E4).

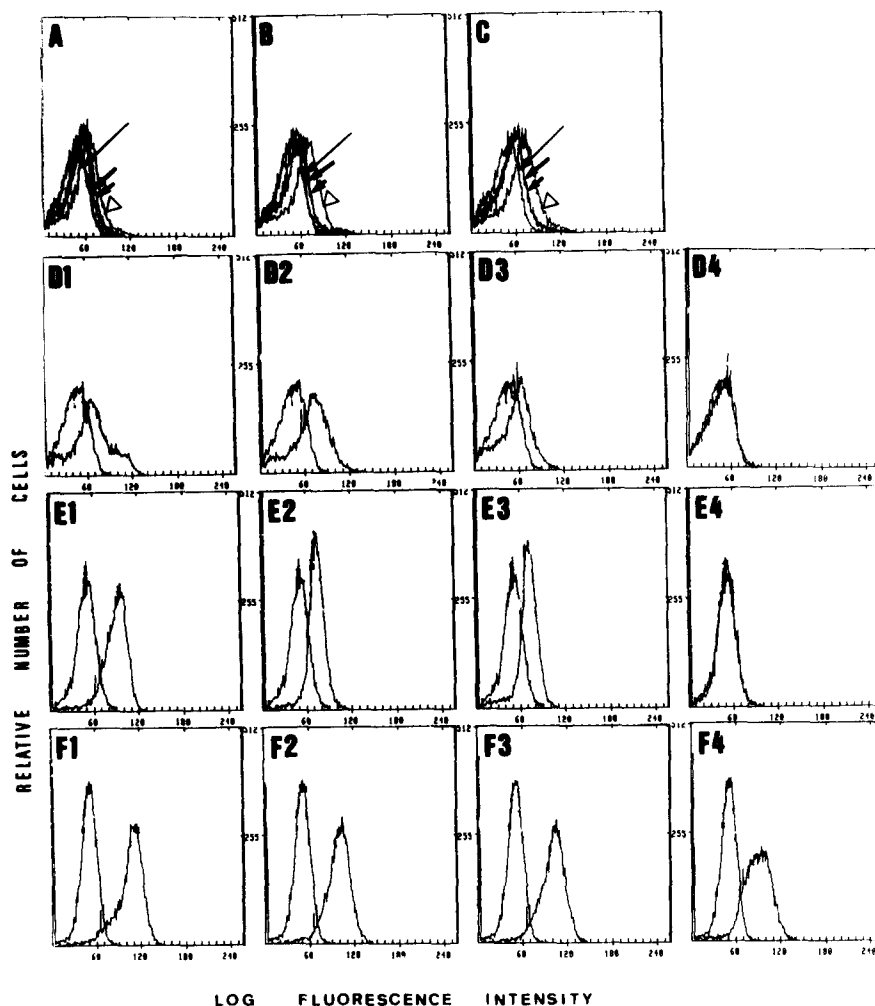
These data indicate that reduced TAP2 expression in Ad12-

**Table 4.** Densitometric Analysis of Peptide Transporters, Class I, and  $\beta_2m$  Expression in Adenovirus-transformed Cells

Cell Line*	TAP1	TAP2	H-2	PD1	$\beta_2m$
	Densitometric units <sup>†</sup> (normalized)				
M1	1.0	1.0	1.0	1.0	1.0
VAD12.42	0.2	0.010	0.3	1.0	0.3
VAD12.79	0.1	0.008	0.3	1.4	0.3
A5O1	12.2	4.3	5.0	5.4	3.0
A5O3	1.7	0.8	1.0	1.9	0.9
A5O5	11.1	5.3	1.0	1.2	1.3
ME1	3.3	1.3	0.5		0.8
ME5	0.9	1.3	1.3		1.3

\* M1 is a normal cell line; VAD12.42 and VAD12.79 are Ad12-transformed cell lines; A5O1, A5O3, and A5O5 are E1Ad5-transformed cell lines; ME1 and ME5 are Ad5-transformed cell lines.

† The results of densitometric scanning of the specific hybridizing signals were first divided to that of actin and then normalized to the value of M1 cells. The same blot was hybridized to all the probes described above.



**Figure 6.** Cell surface expression of class I antigens is reconstituted in TAP2- but not in TAP1-transfected cells. VAD12.79 cells (A) were transfected with PSV2Neo (B), TAP1 (C), and TAP2 (D and E). After transfection, the cells were selected with 800  $\mu\text{g}/\text{ml}$  G418, grown to confluence, and pooled for FACS<sup>®</sup> analyses (B-D), or individual colonies were isolated and expanded in culture. One of the individual colonies transfected with TAP2 is shown in F. Cell surface expression is compared to that of M1 (F). Antibodies used for FACS<sup>®</sup> analyses were: PT85A (anti-PD1) ( $\nabla$ ) in A, B, C and in D1, E1, and F1. 28.14.8 (anti-H-2D<sup>b</sup>) ( $\rightarrow$ ) in A-C, and in D2, E2, and F2. 27.11.13 (anti-H-2D<sup>b</sup>) ( $\longrightarrow$ ) in A-C and in D3, E3, and F3. 20.8.4S (anti-H-2K<sup>b</sup>) ( $\longrightarrow$ ) in A-C and in D4, E4, and F4.



transformed cells partially accounts for the reduced levels of class I expression. However, the failure of TAP2 gene expression to enhance H-2K<sup>b</sup> expression and only partially restore H-2D<sup>b</sup> expression, indicate that other factors are also involved.

## Discussion

MHC class I molecules are a family of highly polymorphic cell surface glycoproteins that have as a primary function the property of binding and presenting foreign peptides to CTL (61–63). After activation, CTL lyse cells that express such peptides. This mechanism is known to be important in controlling pathogenic infections and is also involved in tumor resistance. The lack of cell surface MHC class I proteins in human tumors, as well as in some virus- and carcinogen-induced tumors, undoubtedly interferes with this CTL recognition process (2, 13, 64, 65).

Tumors transformed by the highly oncogenic Ad12 have been used as model systems for demonstrating the direct correlation between tumorigenicity and lack of host immune responses due to suppression of class I expression (20–22). In some of the tumors induced by the virus, downregulation of class I expression was shown to correlate with decreased levels of class I transcription (23–26). However, characterization of a large panel of Ad12-transformed cells in our laboratory revealed that in most cell lines cell surface expression was significantly decreased, whereas the steady state levels of class I and  $\beta_2m$  mRNA were similar or moderately reduced compared to normal cell lines (27). It was further demonstrated that synthesis and transport of class I molecules to the cell surface was inefficient in these Ad12-transformed cell lines (28).

The transport rate of MHC class I molecules through the cell appears to be primarily controlled by their rate of egress from the ER to the *cis*-Golgi compartment (66, 67). The noncovalent interactions of the class I heavy chains with  $\beta_2m$  (40) and peptide (15) combine to stabilize the complex. In cells lacking  $\beta_2m$ , newly synthesized class I heavy chains do not attain a mature structure (68, 69), and are inefficiently transported to the cell surface (38, 40, 70–73). Similar effects were observed in cells with mutated peptide transporter genes that are deficient in ER peptides (10, 15, 16).

The present paper demonstrates that the highly oncogenic Ad12 affects class I antigen levels by downregulating the expression of the peptide transporter genes, thereby leading to reduced availability of relevant peptides for stable assembly and transport of class I molecules to the cell surface. For the Ad12-transformed cells, PD1 expression could be enhanced by incubation at 26°C (Fig. 2), and H-2K and, especially H-2D expression could be enhanced by incubation with relevant peptides (Figs. 3 and 4). Addition of  $\beta_2m$  to the culture media did not induce the expression of class I antigens, and  $\beta_2m$  in combination with micromolar concentrations of peptides did not further enhance the expression of class I antigens. These results support the conclusion that in these cell lines, the limited amount of peptides available for binding to class I molecules contributes to the greatly reduced levels

of expression of these molecules. However, it can not be ruled out that limited amounts of endogenous  $\beta_2m$ , along with possible differences in the affinity of PD1 and H-2 antigens for  $\beta_2m$ , might also contribute to the reduced levels of expression of class I molecules. Thus, if PD1 has a higher affinity than H-2 for a limited supply of  $\beta_2m$  molecules, it would attain a conformation that is transported, albeit more slowly, to the cell surface even in the absence of peptides. This could explain why PD1 expression was enhanced in Ad12-transformed cell lines at 26°C, whereas in general H-2 expression was not. This possibility is further supported by the results obtained with TAP2 transfection. It was shown (Fig. 6) that TAP2 transfection nearly fully reconstituted PD1 expression, partially reconstituted H-2D expression, and failed to reconstitute H-2K expression. Further experiments, including the transfection of  $\beta_2m$  expressing plasmid vectors, are required to determine whether the inability of TAP2 gene expression to fully reconstitute cell surface class I expression is related to limited supply of intracellular  $\beta_2m$ .

The expression of both TAP1 and TAP2 was suppressed in Ad12-transformed cell lines, suggesting that they are regulated by common transcriptional mechanisms. The fact that TAP2 mRNA levels were more markedly decreased by about 10-fold than TAP1 mRNA levels suggests either the presence of multiple suppressive mechanisms or that some factor(s) has a more pronounced effect on the expression of TAP2 than TAP1.

Ad5E1-transformed cell lines expressed higher levels of TAP1 and TAP2 mRNA than the normal cell line. A threefold induction in TAP1 mRNA was also seen in the Ad5-transformed cell line ME1, whereas such an effect was not observed in another Ad5-transformed cell line, ME5. The differences between the cell lines maybe related to the absence of E1a-mRNA in ME5 cells and suggests that Ad5E1a has a transactivating effect on the expression of both TAP1 and TAP2. This conclusion is further supported by the observation that in the Ad5E1-transformed cell A5O3 there is low level of E1a, the expression of TAP1 is activated only by twofold, and the expression of TAP2 is not activated. These observations indicate that gene-specific sequence elements associated with TAP1 and TAP2 can be either up- or downregulated by viral oncogenes.

Peptide transporter and class I genes are not activated or suppressed to the same extent in adenovirus-transformed cells. The difference in TAP1 and TAP2 mRNA levels between Ad5E1- and Ad12-transformed cells ranges between 50 and 100 for TAP1 and more than 500-fold for TAP2, whereas  $\beta_2m$  and H-2 mRNA levels vary by a maximum of 15-fold, and PD1 mRNA level varies by a maximum of 5-fold. Both class I and peptide transporter genes were induced by  $\alpha/\beta$  and  $\gamma$  interferons, but whereas the levels of class I mRNA were only induced 2–4-fold, TAP1 and TAP2 mRNA levels were induced over 100-fold (Winograd et al., manuscript in preparation). These results suggest that different transcriptional mechanisms are likely to be involved in the regulation of class I and peptide transporter genes in this system and emphasize the conclusion that the suppression of peptide trans-

porter gene expression is the critical factor that limits cell surface expression of PD1 and H-2D<sup>b</sup> in Ad12-transformed cells.

The fact that class I-specific peptides did not enhance class I molecule expression in any of the other cell lines, including the Ad5-transformed cell line ME1, which expresses lower levels of class I antigens than ME5, is consistent with normal levels of peptide transporter gene expression in these cells. The relatively low expression of class I antigens in the ME1 cell line is most likely related to the low level of class I mRNA in these cells.

Transfection of TAP1, alone, or in combination with TAP2 did not enhance the expression of class I molecules in an Ad12-transformed cell line. These data clearly indicate that levels of TAP2, and not TAP1, limit the transport of PD1 and H-2D<sup>b</sup> molecules; however, the inability of TAP2 expression to enhance H-2K<sup>b</sup> expression and only to partially reconstitute H-2D<sup>b</sup> expression indicates that a limited peptide supply is not the only explanation for reduced class I expression in these cell lines.

The effects of H-2D<sup>b</sup>- and H-2K<sup>b</sup>-specific peptides were quantitatively different. H-2D<sup>b</sup> molecules were induced to higher levels, and by lower concentration of peptides, as compared with H-2K<sup>b</sup> molecules. There are several possible explanations for this effect: first, the differences between the two class I antigens might indicate that the conformation of H-2K<sup>b</sup> heavy chains is more dependent on  $\beta_2m$  and/or peptide than H-2D<sup>b</sup> heavy chains, leading to less efficient

transport and less accumulation on the cell surface. This possibility is supported by observations that H-2D<sup>b</sup> heavy chains do not require  $\beta_2m$  for cell surface expression (38). Another possibility is that the relatively lower levels of H-2K<sup>b</sup> mRNA leads to a decrease in the number of H-2K<sup>b</sup> molecules available for peptide binding. Since the transfection of the cells by TAP2 leads to the reconstitution of H-2D<sup>b</sup> and PD1 expression but does not affect the expression of H-2K<sup>b</sup> molecules, the latter possibility seems more likely.

Restifo et al. (19) recently identified human cancers with defective antigen processing capacities that do not express TAP1 and TAP2 mRNA. The authors did not determine whether this deficiency is the result of the transformation event, since the extent of the processing capacity and peptide transporter expression in the normal counterparts of these cell lines was not established. Loss of TAP1 and HLA expression was also reported in cervical carcinomas (74). Our results suggest that the suppression of peptide transporter genes may be caused by the transformation event. Moreover, only certain oncogenic viruses seem to have the capacity to cause such suppression. Transformed cells that do not express peptide transporter genes and, as a result, do not transport class I molecules, may undergo selection in vivo and develop into nonimmunogenic tumors with oncogenic potential. This system provides a model for evaluating the roles of various factors in the class I biosynthetic pathway in normal and malignant cells.

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