LMP-associated Proteolytic Activities and TAP-dependent Peptide Transport for Class I MHC Molecules Are Suppressed in Cell Lines Transformed by the Highly Oncogenic Adenovirus 12

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Summary

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 in vivo oncogenicity of this virus. In primary embryonal fibroblasts (H-2^b) that express a transgenic swine class I antigen (PD1), Ad12-mediated transformation results in inhibition in transport of newly synthesized class I molecules, as well as significant reduction in transporter associated with antigen presentation (TAP) gene expression. In this report we show that reexpression of TAP molecules either by stable transfection of mouse TAP genes or by infection with recombinant vaccinia viruses expressing human TAP genes, only partially reconstitutes the expression and transport of the class I molecueles. Further analysis of Ad12-transformed cells revealed that the expression of both LMP2 and LMP7, but not of other proteasome complex components, was downregulated, resulting in altered proteolytic activities of the 20S proteasomes. Reconstitution of both TAP and LMP expression resulted in complete restoration of PD1 cell surface expression and enhanced expression of the endogenous H-2D^b molecules. Despite high expression of TAP, LMP, and class I MHC molecules encoded by recombinant vaccinia viruses, in reconstituted Ad12-transformed cells, efficient transport of H-2 class I molecules could only be achieved by treatment of the cells with γ-interferon. These data suggest that an additional factor(s) that is interferon-regulated plays a role in the biosynthetic pathway of the class I complex, and that its function is deficient in this cell system. Thus, Ad12 viral transformation appears to suppress the expression of multiple genes that are important for antigen processing and presentation, which allows such transformed cells to escape immune surveillance. This coordinate downregulation of immune response genes must likely occur through their use of common regulatory elements.

The MHC class I genes play key roles in numerous immunological processes involving the presentation of "foreign" antigens for recognition by CTL (1). By this mechanism, the immune system is able to control infectious diseases and the growth of tumor cells. Indeed, tumors of various origin have been shown to express low levels of class I antigens, a characteristic that may contribute to their escape from immune surveillance (2–5). Transfection of cell lines by class I MHC genes did not always result in the expression of class I molecules on the cell surface although increased levels of class I mRNA were observed (6). Thus, the expression of class I MHC molecules may be suppressed during transcription of the genes or during synthesis, assembly, and transport of molecules to the cell surface.

The biochemistry and cell biology of antigen processing and presentation by class I MHC molecules have been analyzed in detail in the past six years (7, 8). It is well established that the efficient transport of class I molecules to the cell surface depends on assembly of the heavy chain/β2m dimer with peptides. Such peptides, generated by cleavage of proteins in the cytosol, are actively transported by a heterodimeric complex known as transporter associated with antigen presentation (TAP)[1], into the endoplasmic reticulum (ER), where assembly with class I molecules takes

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[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; TAP, transporter associated with antigen presentation.

place. The trimeric complex is then transported through the Golgi apparatus to the cell surface. 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 for presentation to CTL (9–11).

The function of the peptide transporters was determined by analysis of class I assembly, transport, and antigen presentation in selected TAP-negative cell lines, and in TAP knockout mice. Tumor cell lines with deletions in the MHC locus encompassing TAP and proteasome-associated LMP genes (such as T2 and LBL 721.174), or with a TAP mutated gene (the murine RMA/S), have drastically reduced cell surface levels of class I molecules, and are defective in class I transport and in antigen presentation (7, 8, 12, 13). Splenocytes from TAP1 knockout mice reveal similar characteristics (14). Expression of class I molecules and antigen presentation can be restored by expressing TAP1 and TAP2 in these mutant cells (15, 16), indicating the indispensability of these molecules for class I expression and antigen presentation. The proteases involved in the generation of peptide antigens have not been clearly identified, although attention has focused on the multicatalytic proteinase complex, or proteasome (17). This protease consists of homologous subunits of 20–35 kD, and cleaves peptides and proteins after hydrophobic, basic, and acidic residues. Several lines of evidence are consistent with the proteasome's role in antigen presentation. These include the effects of protease inhibitors on the generation of peptides presented by class I molecules (18), and the involvement of proteasome-mediated ubiquitin-dependent proteolysis in antigen presentation (19). The two proteasome subunits, LMP2 and LMP7, are encoded by genes located in the MHC and are closely linked to the TAP genes (20–22). Transcription of LMP genes is induced by γ-interferon and is followed by their incorporation into the proteasomes, reorganization of the proteasome subunits, and modulation of the proteolytic activities and cleavage site preferences of the 20S proteasomes (23–27). Thus, LMP molecules may play a role in controlling the production of peptides that are more compatible to class I binding. Indeed, mice lacking the LMP7 component appear to have a partial defect in HY antigen presentation, and lower levels of certain class I alleles (28). In addition, LBL 721.174 cells reconstituted with TAP1 and TAP2, but deleted of a sequence containing the LMP genes, still demonstrate selective deficiency in the presentation of certain antigens by HLA A2 and H-2D^b (29). Moreover, the ability to present efficiently HLA A2–specific peptides could be restored by expressing the LMP7 gene product (29), implying a role for LMP molecules in supplying selective sets of peptides.

Human tumors and tumor cell lines exhibiting defects in transport of class I molecules and in antigen presentation have been recently described (30–34). A large number of Burkitt's lymphoma cell lines are unable to process intracellular antigenic determinants, and are highly resistant to class I–restricted CTL-mediated lysis (33, 35). Khanna et al. (33) demonstrated that both peptide transporters (TAP1 and TAP2) are downregulated in the tumor cells, resulting in inefficient transport of peptides to the ER. Downregulation of class I MHC expression has been found also in a substantial number of cervical carcinomas containing human papilloma virus 16 (34). Cromme et al. (32, 34) demonstrated that in neoplastic cervical cells that had lost their class I MHC surface expression, the peptide transporter TAP1 was no longer expressed by some or all of the tumor cells (36). Some DNA viruses are associated with both human and murine tumors that are deficient in class I molecule transport; cervical carcinomas harbor human papilloma virus sequences (34), Burkitt's lymphomas contain EBV sequences (35), and in Ad12-transformed rodent cells the early region oncogenes (E1A and E1B) of Ad12 are essential and sufficient for Ad12-mediated transformation (37). We previously showed that reexpression of the TAP2 gene in an Ad12-transformed cell line, which is downregulated for the expression of TAP genes, partially restored PD1 (a miniature swine class I transgene) and H-2D^b expression, but did not reconstitute that of the endogenous H-2K^b (31). These results raised the possibility that transformation by Ad12 and possibly other DNA viruses affects the expression of class I molecules not only by suppressing peptide transporter genes, but also of other genes encoding molecules involved in the assembly and transport of class I molecules.

We now show that the reexpression of TAP genes in Ad12-transformed cells is not sufficient for efficient transport of class I molecules. This inhibition is coordinately regulated with deficiencies in specific sets of peptides and the complete knockout of LMP genes in this system. Furthermore, we suggest the existence of an additional IFN-regulated factor(s) which plays a role in the biosynthetic pathway of the class I complex and its function is deficient in these 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 (38). The mice, which are homozygous for the transgene, were bred at the Tel Aviv University breeding facility.

Cell Cultures, Stable Transfections, and IFN Treatment. The Ad12-transformed (VAD12.79, VAD12.42, VAD12.20, VAD12.25, VAD12.36), Ad5-transformed (ME1), E1Ad5-transformed (A5O1, A5O5), one of the TAP-transfected cell lines (C1.1), and the normal cell line (M1) have been previously described (31, 38). Additional TAP-transfected cell lines are described in the text. PD1.1 is a RadLV-induced thymic leukemia expressing high levels of class I antigens (39).

Stable transfections were done by the calcium phosphate-DNA coprecipitation method as previously described (31) with the following modifications: the transfection cocktail contained 5 μg of pSG5 plasmids encoding LMP2, or LMP7 cDNAs, or both, and 1 μg of the plasmid pBabe encoding the puromycin resistance gene (a kind gift from P. Murray, Whitehead Institute, Boston, MA). 24 h after transfection the cells were washed with PBS and 24 h later the media were supplemented with 2 μg/ml of puromycin (Sigma Chemical Co., St. Louis, MO).

Cell lines were treated with γ-IFN (100 U/ml) (Boehringer

Mannheim, Mannheim, Germany) 48 h before harvesting or before infection with recombinant vaccinia viruses. The cell lines were maintained in DMEM supplemented with 2 mM glutamine, 10% FCS, penicillin, streptomycin, gentamycin, and amphotericin B at the recommended concentrations (40). Media and supplements were purchased from Biological Industries (Bet Ha'emek, Israel).

Recombinant Vaccinia Viruses and Infection. Recombinant vaccinia viruses expressing H-2K^b and H-2D^d (41), human TAP1 and TAP2 (42), and the hybridoma TW2.3 which secretes antibodies directed against a vaccinia-encoded protein (43) were kindly supplied by J. Yewdell (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Infections with recombinant vaccinia viruses and immunoprecipitations were carried out as previously described (30), with the following modifications: cells were infected with 10 multiplicity of infection/cell for 60–90 min, washed with PBS, and incubated in methionine-free medium for 15 min. Cells were then labeled for 30 min and immunoprecipitations were carried out as described. Antibodies directed against the vaccinia internal protein were used to monitor efficiency of infection.

Probes and Plasmids. Actin probe is a PstI-PstI fragment from chicken β actin cDNA cloned in pBR322 (44); the TAP1 and TAP2 probes are XbaI-HindIII and KpnI-KpnI fragments containing TAP1 and TAP2 cDNAs, respectively, cloned in pcDNAI Neo (Invitrogen, San Diego, CA); the LMP2 and LMP7 probes are EcoRI-EcoRI fragments containing cDNAs cloned in pGEM (Promega Corp., Madison, WI). The TAP- and LMP-containing plasmids were a kind gift from Dr. J. J. Monaco (University of Cincinnati, Cincinnati, OH) (45).

Antibodies. The following antibodies were used for FACS® analyses and immunoprecipitations: 20.8.4S (recognizes α1/α2 epitope on H-2K^b) (46); 28.14.8 (recognizes an epitope on α3 domain of H-2D^b heavy chains with or without associated β_2m) (46–48); B22.249 and 27.11.13 (recognize an α1 epitope and a public conformation-dependent epitope, respectively, on H-2D^b) (46); 34.2.12 (recognizes an epitope on the α3 of H-2D^d) (48); 34.5.8 (recognizes an epitope on the α1/α2 domain of H-2D^d) (49). PT85A (recognizes a public determinant on swine lymphocyte antigens) (50). Affinity-purified antibodies directed against LMP2, LMP7, and MC3 (51, 52) were used for Western analyses and immunoprecipitations. FITC-conjugated goat anti–mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

FACS® Analysis. Cells were harvested by mild trypsinization, followed by washes in media supplemented with 5% FCS and 0.01% sodium azide. About 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. Cells were washed with PBS and fluorescence intensity and analyzed with a cell sorter (Becton Dickinson & Co., Mountain View, CA).

RNA Analysis. Cytoplasmic RNA was prepared using a modification of the White and Bancroft method (53) as previously described (31). RNA was denatured and run on a 1.2% formaldehyde/formamide agarose gel, blotted onto a Hybond-N membrane (Amersham International, Little Chalfont, UK) and hybridized with the appropriate probe, which had been labeled with [α-^{32}P]dCTP (Rotem Industries, Dimona, Israel), using a random priming labeling kit (United States Biochemical Corp., Cleveland, OH).

The hybridization solution contained 4 × SSC, 50% formamide, 0.2% SDS, 0.1% polyvinylpyrrolidone and 100 μg/ml sheared salmon sperm DNA. Hybridizations were carried out at 42°C followed by washes with 2 × SSC, 0.1% SDS at room temperature, and 0.2 × SSC at temperatures ranging between 55 and 65°C. RX x-ray film (Fuji, Tokyo, Japan) was exposed to the blot and the resulting bands were scanned with a densitometer. After stripping with a boiling solution of 0.1% SDS, the blot was used for additional hybridizations.

Purification of 20S Proteasomes. Frozen pellets of 5 × 10^8 A505 or VAD1279 cells were lysed and 20S proteasomes were purified as described (51). A purity of >90% was obtained as judged by Coomassie staining. Native proteasome protein was quantified by UV absorption at 280 nm using an extinction coefficient of 1.0 cm^2/mg. The yield was 150 μg proteasome/5 × 10^8 cells.

Protease Assays and HPLC Separation. Fluorogenic substrate peptides Succ-LLVY-MCA, Z-GGL-MCA, VGR-MCA (Bachem, Heidelberg, FRG) were prepared from 10-mM stocks in DMSO and incubated at several final concentrations for 1 h at 37°C with 100 ng purified proteasome in 1 ml buffer containing 50 mM Tris/HCl, pH 7.5, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA. The reaction was terminated by the addition of 100 μl buffer containing 30 mM NaAc, pH 4.3, 100 mM $CH_2ClCOOH$, and 70 mM acetic acid. Fluorescence was determined with a spectrofluorometer (RF-5001 PC; Shimadzu, Duisburg, Germany) at 380 nm excitation/440 nm emission. The Z-LLE-βNA substrate was freshly dissolved for each experiment, and the reaction was terminated by the addition of 1 vol ethanol. Measurements were performed at 335 nm/410 nm.

Digestion of a synthetic 25-mer peptide derived from the sequence of murine CMV pp89 IE protein was carried out as previously described (27). A quantity of 20 μg polypeptide (kindly provided by Dr. P. Henklein, Humbolldt University, Berlin, Germany) was dissolved in 300 μl buffer containing 20 mM Hepes/KOH (pH 7.8), 2 mM $MgAc_2$, 1 mM DTT, and digested with 1 or 2 μg purified proteasomes for 24 h at 37°C. Cleavage products were analyzed by reverse phase HPLC: 50 μl of the digest were applied to a 4.6 × 250–mm Ultrasphere RP18 column using equipment from Beckman (Munich, Germany). Products were eluted at a flow rate of 0.5 ml/min with solutions A (water/0.1% TFA) and B (acetonitrile/0.1% TFA): 0–5 min, 0% B; 5–40 min linear increase to 60% B. Peaks were detected at 220 nm.

Metabolic Labeling and Immunoprecipitation. Cells were grown to 80% confluence and starved for 30 min in methionine-free medium. They were labeled in methionine-free medium containing 100–250 μCi/ml [^{35}S]methionine (Amersham International) for 30 min, washed with PBS, and chased for the indicated intervals. Immunoprecipitation of LMP2 and LMP7 has been previously described (54). Immunoprecipitation of class I molecules was carried out using the same procedure with the following modification: the lysis buffer contained 0.5% Triton X-100, 50 mM Tris, pH 7.5, and 150 mM NaCl. The immunoprecipitates were washed with buffer containing 0.1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl. For endo H treatment, immunoprecipitates were eluted by adding 25 μl of 50 mM Tris, pH 8, 1% SDS, and boiling for 5 min. The samples were centrifuged and the supernatant was added to 25 μl of 50 mM citrate buffer, pH 5.5, containing 500 U of endo H (New England Biolabs, Beverly, MA). The samples were incubated at 37°C for 18 h, followed by the addition of sample buffer. All the buffers contained the following protease inhibitors; 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma Chemical Co., St. Louis, MO). The pre-

cipitates were fractionated on 10–15% SDS-PAGE and X-OMAT AR x-ray films (Eastman Kodak Co., Rochester, NY) were exposed to the dried gels. Bands were scanned with a densitometer.

Western Blot Analysis. Various amounts of protein from total cell extracts (200 μg) or purified proteasomes (5.0 μg, 1.0 μg, 0.2 μg, 0.04 μg), prepared from "normal," E1Ad5-transformed, and Ad12-transformed cells, and LMP-transfected VAD12.79 cells, were fractionated on 15% SDS-PAGE and blotted in 25 mM Tris/HCl (pH 8.8) + 0.19 M glycine onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Blots were blocked for 1 h in buffer containing PBS, 5% (wt/vol) milk powder, 10% horse serum, and 0.4% Tween 20, and incubated overnight at 4°C with antisera directed against LMP2 (50), LMP7 (50), and a constitutive proteasome subunit, MC3 (51), all diluted 1:200 in PBS/0.4% Tween 20/2% milk powder. Blots were washed three times in PBS/0.4% Tween 20 and incubated for 1 h in goat anti–rabbit peroxidase conjugate (Immunopräperate Berlin, Berlin, Germany) diluted 1:1,000 in PBS/0.4% Tween/2% milk powder. The blots were washed four times and x-ray films were exposed to the enhanced chemiluminescence–treated blots (Boehringer Mannheim).

Nonequilibrium pH Gradient Electrophoresis/PAGE Two-Dimensional Gels. TCA precipitates containing 50 μg proteasomes were agitated overnight in 60 μl NEPHGE sample buffer containing 9.5 M urea, 2% NP-40, 5% ampholines (pH 3–10, Servalyt; Serva, Heidelberg, Germany), 0.3% SDS, and 5% β-mercaptoethanol. Samples were applied to the gel and run for 4 h at 400 V. After 45 min equilibration in 25 ml buffer containing 10% glycerol, 10% β-mercaptoethanol, 2.3% SDS, 90 M Tris/HCl, pH 6.8, the rod was applied to a 15% SDS-PAGE with SDS sample buffer containing 1% agarose. The proteins were analyzed by Coomassie staining.

Results

Cell Surface Expression and Transport of Class I Molecules in an Ad12-Transformed Cell Line Stably Transfected with TAP1, TAP2, and TAP1+TAP2 Expression Vectors. We previously characterized a panel of Ad12-transformed cell lines expressing very low levels of class I antigens, comparing them with E1Ad5-transformed and normal fibroblast cell lines (38, 55). We found that the expression of both the endogenous class I genes (H-2K^b and H-2D^b), as well as that of a miniature swine class I transgene (PD1), were suppressed. The reduced levels of H-2D^b and PD1 resulted mainly from their inefficient transport to the cell membrane, a phenomenon that could be explained by the limited amounts of peptides present in the ER, and a 100-fold reduction in the steady state level of TAP2 mRNA (31). However, reexpression of TAP2 in an Ad12-transformed cell line (VAD12.79) resulted only in partial reconstitution of H-2D^b cell surface expression, while the expression of PD1 was comparable with that of the normal cell line (31). Fig. 1 *A* compares the cell surface expression of a TAP2-transfected clone with that of a TAP1-transfected clone, and TAP1 + TAP2-transfected clones. The FACS® analysis shows that TAP1 transfection did not affect the expression of class I antigens, but coexpression of TAP1 and TAP2 enhanced by fourfold the cell surface expression of H-D^b and by four- to sevenfold the expression of PD1 as compared to cells transfected with TAP2 only. While the expression of PD1 was comparable or even exceeded that of the normal cell line M1, the expression of H-2D^b was fourfold lower. Fig. 1 *B* shows the steady state level of TAP1 and TAP2 mRNA in mixed populations of TAP1- and TAP1+TAP2-transfected cells (lanes *4* and *5*, respectively), and randomly selected TAP-transfected clones, expressing either TAP1 (Cl.5), TAP2 (Cl.1), or both (Cl.7 and Cl.9). As depicted in the figure the steady state level of TAP1 and TAP2 mRNA in the TAP-transfected cell lines was significantly higher than in the normal cell line M1 or the E1Ad5-transformed cell line A5O5, indicating that the level of TAP mRNA is not limiting in the transfected cells.

To further locate the defect in TAP-transfected cells, egress of class I molecules from the ER was analyzed by their endo H resistance acquisition in pulse-chase experiments (Fig. 2). The transport rate of PD1 (*A*) and H-2D^b (*B*) was studied in M1, VAD12.79, and TAP-transfected cell lines. To differentiate between empty and peptide-loaded class I molecules, antibodies directed against an epitope on the α1 domain (B22.249) (Fig. 2 *C*) or on the α3 domain (28.14.8) of H-2D^b (Fig. 2 *B*) were used for immunoprecipitation. The results show (Fig. 2, *A* and *B*) that the rate of endo H resistance acquisition of PD1 was indeed comparable in the TAP-transfected and in the normal cell lines, and that the observed reduction in cell surface expression of H-2D^b was directly correlated with the inefficient transport of these molecules through the Golgi apparatus. However, 10–20% of the H-D^b molecules (Fig. 2 *C*, *inset*), which were recognized and immunoprecipitated by the conformation-dependent α1 antibody (B22.249), acquired endo H resistance in TAP-transfected cells (Fig. 2 *C*). These H-2D^b molecules presumably accumulate on the cell surface and are detected in FACS® analysis. These data, in conjunction with the results of the FACS® analysis of TAP-transfected Ad12-transformed cells, support the notion that although expression of TAP2 in Ad12-transformed cells is essential, additional factors are necessary for obtaining efficient transport and maximal cell surface expression of the endogenous class I molecules.

TAP-independent Inhibition of Class I Molecule Transport in Ad12-transformed Cells. The kinetics of assembly and transport of the class I complex depends on the relative amounts of the heavy chains and β2m (56, 57). The likelihood of limited amounts of β2m interfering with stable assembly and transport of the class I complex was ruled out in view of the finding that the expression of high levels of β2m in TAP-transfected Ad12-transformed cells via recombinant vaccinia viruses, or the addition of recombinant β2m to cell extracts, did not enhance assembly or transport of H-2D^b (data not shown). To exclude the possibility that endogenous class I molecules were being produced in limiting amounts, we analyzed the transport of H-2D^d produced by recombinant vaccinia viruses. As can be seen in Fig. 3 *A*, there was inefficient transport of H-2D^d molecules through the Golgi in VAD12.79 and in TAP-transfected VAD12.79 cells. These results are comparable with the results obtained for the endogenous H-2D^b. Moreover, when VAD12.79 was infected with recombinant vaccinia

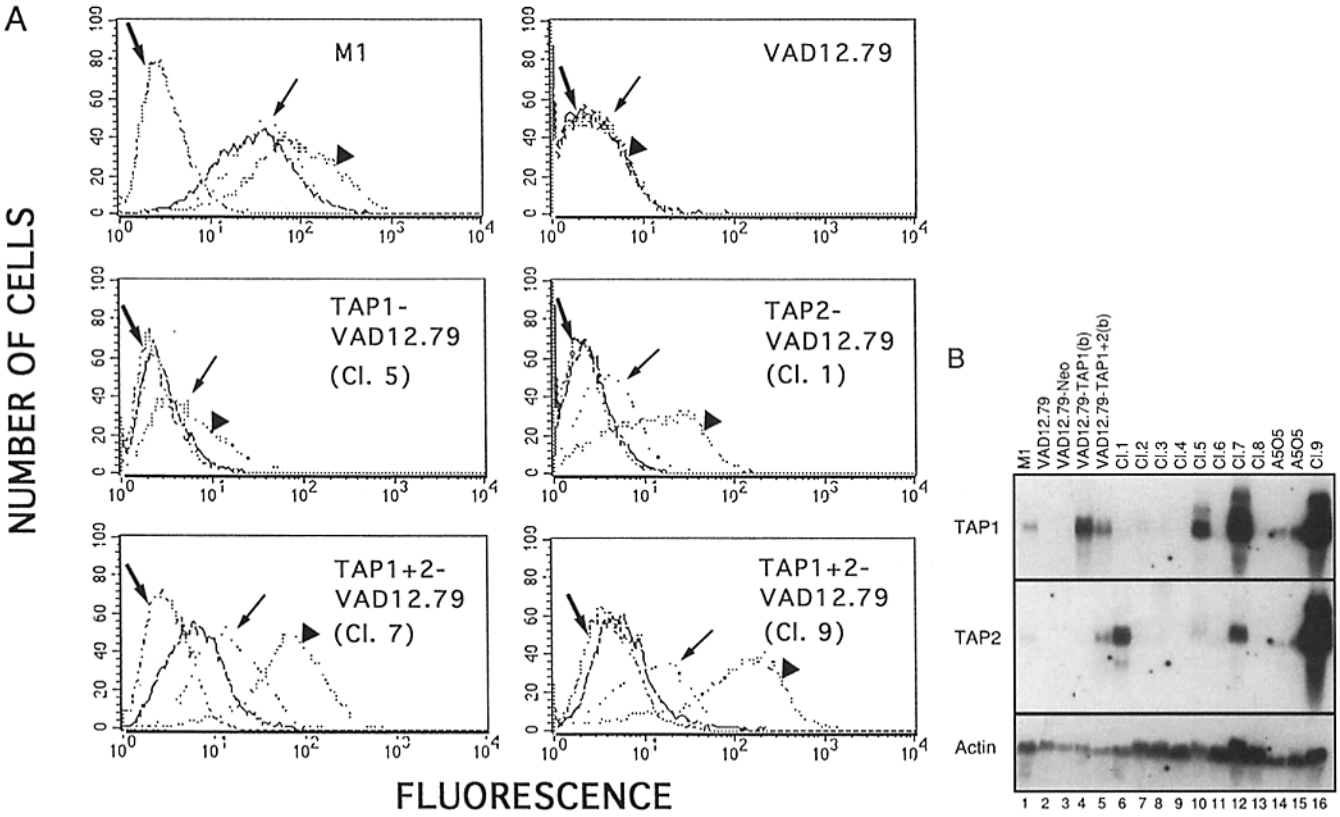

Figure 1. Expression of class I antigens in TAP-transfected Ad12-transformed cell lines. (*A*) Cell surface expression of class I antigens on the normal cell line (*M1*), Ad12-transformed cell line (*VAD12.79*), TAP1-transfected VAD12.79 (*Cl.5*), TAP2-transfected VAD12.79 (*Cl.1*), TAP1+2-transfected VAD12.79 (*Cl.7, Cl.9*) was analyzed by immunofluorescence staining with the following antibodies: PT85A (anti-PD1) (*arrowhead*), 20.8.4S (anti-H-2K^b) (—), and 27.11.13 (anti-H-2D^b) (*narrow arrow*). Second antibody control (*fat arrow*). Cl, individual clone. (*B*) Northern analysis of mRNA from TAP-transfected VA12.79 and control cell lines (*M1, A5O5, VAD12.79, VAD12.79-Neo*) was carried out using 20 μg of cytoplasmic RNA which was fractionated on a formaldehyde/formamide agarose gel, transferred to nylon membrane, and hybridized as described in Materials and Methods with probes specific for TAP1, TAP2, and actin. *VAD12.79-TAP1(b), VAD12.79-TAP1+2(b)*: mixed cell populations transfected with TAP1 and TAP1+TAP2, respectively. C1, individual clone.

viruses expressing the human TAP1+TAP2 genes, the transport of PD1 was reconstituted (Fig. 3 *B*) but there was no change in the transport of H-2D^d molecules expressed from a coinfected recombinant vaccinia virus (data not shown).

To determine whether H-2 molecules can acquire endo H resistance, and to rule out any negative effect on transport of endogenous proteins possibly caused by the vaccinia virus infection, cells were treated with γ-IFN before vaccinia infection. Since H-2K^b gene transcription is suppressed in these cells, recombinant vaccinia viruses that encode either H-2K^b or H-2D^d were used for infection. Fig. 4 demonstrates that γ-IFN treatment resulted in two- to threefold enhancement of H-2 transport, so that the rate of acquisition of endo H resistance was comparable with that observed in E1Ad5-transformed cells. Comparable results were obtained with cells pretreated with γ-IFN and infected with recombinant vaccinia viruses expressing H-2K^b or H-2D^d molecules (Fig. 4, *A* and *B*).

Since these results indicated that infection with vaccinia viruses for 60–90 min does not generally interfere with transport of class I molecules, it is conceivable that there is a selective inhibition in the transport of H-2 class I molecules, but not of PD1, in TAP-expressing Ad12-transformed cells. The results also suggest the involvement of a factor(s) that is regulated by γ-IFN and can induce the transport of these molecules. Since γ-IFN induces LMP transcription and thus affects the production of selective sets of peptides (23–27), we next addressed the level of proteasome-associated LMP2 and LMP7 subunits and the proteolytic activities mediated by purified 20S proteasomes prepared from these cell lines.

Expression of LMP2 and LMP7 Molecules in Ad12- and E1Ad5-transformed Cell Lines. To determine the steady state level of LMPs and their inducibility by γ-IFN, as well as the level of proteasome-associated LMPs in Ad12-transformed cells, both crude cell extracts (which may also contain unprocessed proteasome precursors [58]), and purified 20S proteasomes were fractionated on SDS gels and hybridized with antibodies specific for LMP2 or LMP7. Whereas LMP2 and LMP7 were expressed in the crude cell extracts and in purified 20S proteasomes from normal and E1Ad5-transformed cell lines, they were hardly detectable in similar preparations from Ad12-transformed cell lines (Fig. 5, *A* and *B*). Nonetheless, γ-IFN induced the expression of

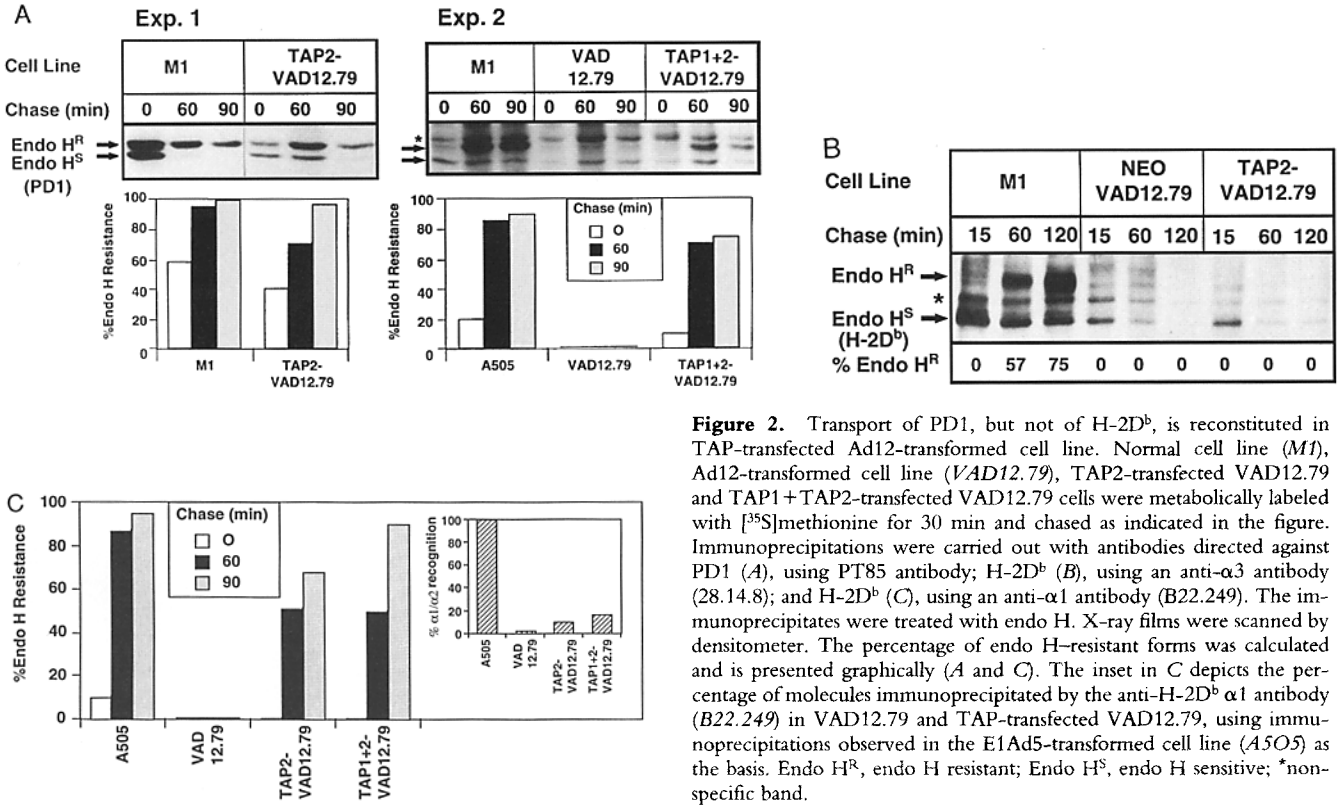

Figure 2. Transport of PD1, but not of H-2D^b, is reconstituted in TAP-transfected Ad12-transformed cell line. Normal cell line (*M1*), Ad12-transformed cell line (*VAD12.79*), TAP2-transfected VAD12.79 and TAP1+TAP2-transfected VAD12.79 cells were metabolically labeled with [^{35}S]methionine for 30 min and chased as indicated in the figure. Immunoprecipitations were carried out with antibodies directed against PD1 (*A*), using PT85 antibody; H-2D^b (*B*), using an anti-α3 antibody (28.14.8); and H-2D^b (*C*), using an anti-α1 antibody (B22.249). The immunoprecipitates were treated with endo H. X-ray films were scanned by densitometer. The percentage of endo H–resistant forms was calculated and is presented graphically (*A* and *C*). The inset in *C* depicts the percentage of molecules immunoprecipitated by the anti-H-2D^b α1 antibody (*B22.249*) in VAD12.79 and TAP-transfected VAD12.79, using immunoprecipitations observed in the E1Ad5-transformed cell line (*A5O5*) as the basis. Endo H^R, endo H resistant; Endo H^S, endo H sensitive; *nonspecific band.

LMP2 and LMP7 in Ad12-transformed and in the TAP-transfected Ad12-transformed cell lines (Fig. 5 *A*). In γ-IFN–treated cells the level of LMP2 was comparable to that observed in untreated M1 and A5O5 cells, while the level of LMP7 was fourfold lower. In addition, an additional band which may be an LMP precursor could be detected in crude extracts from γ-IFN–treated cells. Since application of purified 20S proteasomes (Fig. 5 *B*) allowed us to cover a protein range of >100-fold, the results clearly indicate that the Ad12-transformed cell line, VAD12.79, contains >100-fold less LMP2 and LMP7 than the E1Ad5-transformed cell line A5O5. On the other hand, the level of MC3, which is a constitutive proteasome α subunit (Fig. 5 *B*) and of the δ subunit (visualized by cross-reaction of anti-LMP2 antibodies with the δ subunit), were identical in both Ad12 and E1Ad5-transformed cell lines.

Assuming that the unidentified bands observed in the hybridization with anti-LMP2 and anti-LMP7 antibodies (marked by stars in Fig. 5 *A*) might be LMP precursors produced both in VAD12.79 and A5O5, but not processed to the mature protein in VAD12.79, pulse-chase experiments were carried out using antibodies against LMP7 to immunoprecipitate LMP7 and LMP7 precursors. The assays revealed that the 30-kD LMP7 precursor was being synthesized by A5O5 but not by VAD12.79. Processing of LMP7 by A5O5 was normal, as the precursor was chased into the mature 23-kD after 4 h (data not shown).

The dramatic differences between LMP levels in Ad12 and E1Ad5-transformed cell lines prompted us to investigate whether the level of other components of the 20S proteasome also varied. A two-dimensional PAGE of 20S proteasomes from VAD12.79 and A5O5 is presented in Fig. 6. As can be seen, all the 20S proteasome subunits, aside from LMP2 and LMP7, were expressed in equal amounts in the Ad12-transformed cell line VAD12.79 and the E1Ad5-transformed cell line A5O5. In A5O5, the distribution of the replaceable subunits was 16% LMP2/84% δ and 34% LMP7/66% MB1. These results are identical with those obtained in another fibroblast cell line, B8 (data not shown).

Downregulation of LMP2 and LMP7 in Ad12-transformed Cells. Since the level of LMPs can be regulated both at the transcriptional and at the posttranscriptional levels (51), the steady state level of LMP2 and LMP7 was determined in a panel of Ad12-transformed cells (Fig. 7). The steady state level of both LMP2 and LMP7 mRNA was undetectable in most Ad12-transformed cell lines and reduced by fourfold in VAD12.20. The steady state level of both LMP2 and LMP7 mRNA was 20-fold lower in Ad5-transformed and normal fibroblast cell lines (ME1, A5O5, A5O1, M1) than in a T lymphoma cell line (PD1.1). These results are in full agreement with the Western blot analysis and the immunoprecipitation data, which revealed that both the LMP precursors and the LMPs are absent from Ad12-transformed cells.

Proteasomal Peptide Hydrolyzing Activities of 20S Proteasomes from VAD12.79 and A5O5. Since secondary modifications and the exchange of subunits in the proteasome, as observed in γ-IFN–treated cells, often results in changes in enzymatic properties (23–27), we explored the in vitro en-

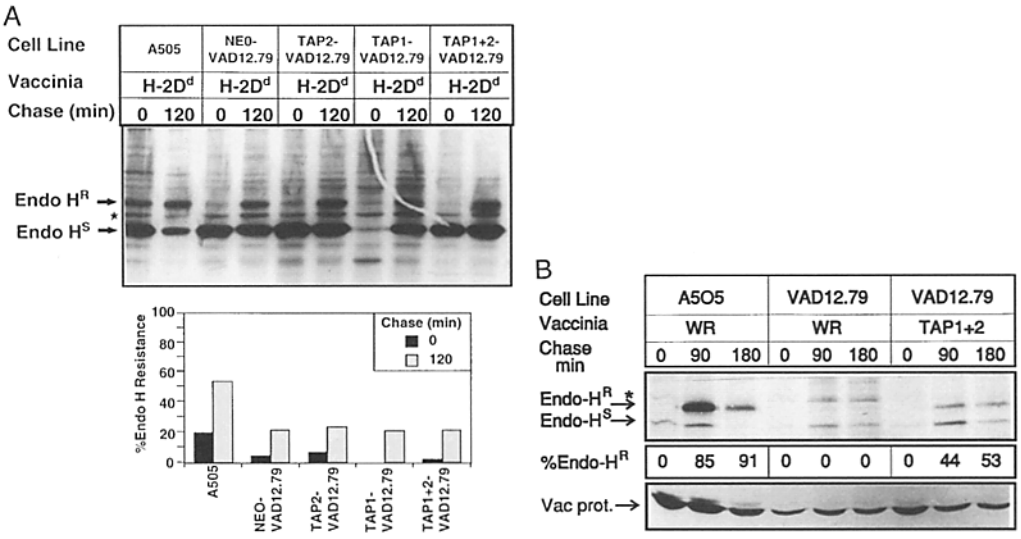

Figure 3. Transport of H-2D^d molecules encoded by recombinant vaccinia virus is inefficient in TAP-transfected and in VAC-TAP–infected Ad12-transformed cells. TAP-transfected cell lines and control cell lines (*A5O5* and *VAD12.79*) were infected for 60 min (*B*) or 90 min (*A*) with recombinant vaccinia viruses expressing H-2D^d (*A*) or human TAP1+TAP2 (*B*) or with wild-type vaccinia virus (*WR*) (*B*). After pulsing, the cells were chased, and class I molecules were immunoprecipitated and treated with endo H as described in the legend to Fig. 2. The following antibodies were used for immunoprecipitation; anti-H-2D^d α3 (34.2.12) in *A*, anti-PD1 (PT85) in *B*, anti-VAC protein in *B*. X-ray films were scanned by densitometer, and the percentage of endo H–resistant forms was calculated and is presented graphically in *A*. The absence of signal in TAP1-VAD12.79 at 0 min chase was due to technical problem with vaccinia infection. Endo H^R, endo H resistant; Endo H^S, endo H sensitive; *nonspecific band.

zymatic activities of purified 20S proteasomes from the Ad12- and E1Ad5-transformed cell lines. The chymotrypsin-like and the trypsin-like activities were assayed during standard fluorogenic peptide substrates with Tyr, Arg, Leu, and Glu at the P1 position. Fig. 8 clearly demonstrates that the cleavage of Leu and Tyr peptides (the chymotrypsin-like activity), but not of Arg (the trypsin-like activity), was lower in A5O5 than in VAD12.79. Comparable reduction in chymotrypsin-like activity was documented in mouse embryonal fibroblasts after treatment with γ-IFN and incorporation of LMP2 and LMP7 into the proteasome (27). These data suggest that transformed cells expressing very low levels of LMPs may differ in their capacity to generate constitutively certain sets of peptides.

Distinct Cleavage Site Preference of 20S Proteasomes from VAD12.79 and A5O5. Although fluorogenic peptide substrates are suitable for characterizing different proteolytically active sites of latent and active 20S proteasomes, they provide little information on how proteasomes react with larger polypeptides which represent in vivo substrates. To determine the capacity of VAD12.79 and A5O5 to hydrolyze a larger polypeptide, the synthetic pp89 25-mer polypeptide, which harbors an antigenic nonamer peptide (27), was subjected to cleavage by purified proteasomes from these cell lines. After complete substrate turnover, the processing products generated from the 25-mer polypeptide by incubation with 20S proteasomes were separated on reverse phase HPLC (Fig. 9). A comparison of the chromatographs indicates that the absence of LMP subunits caused a significant quantitative difference in at least some fragments (RT25.71, 27.81, 29.53, 33.19, 34.44). It is clear that the cleavage specificity of the proteasomes in both cell lines was identical although in VAD12.79 there was a higher concentration of the most prominent peptide (RT33.19), and reduced amounts of at least three other peptides. The same results were obtained whether 1 or 2 μg proteasome preparation was used, indicating that digestion was complete and that the observed differences were not due to incomplete hydrolysis of the 25-mer polypeptide substrate. The results of both enzymatic assays demonstrate, there-

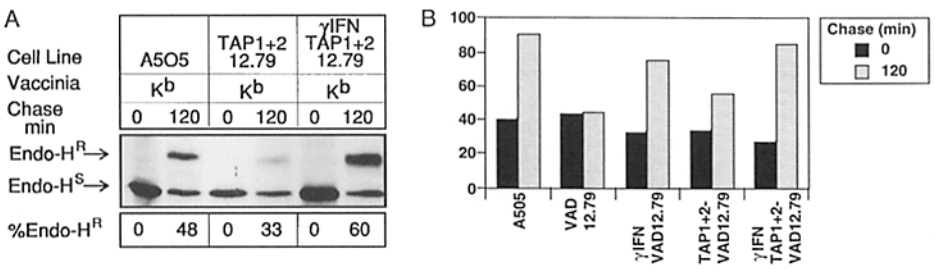

Figure 4. Transport of H-2D^d and H-2K^b encoded by recombinant vaccinia viruses is enhanced after pretreatment of Ad12-transformed cells with γ-IFN. TAP-transfected cell lines and control cell lines (*A5O5* and *VAD12.79*) were infected for 60 min (*A*) or 90 min (*B*) with recombinant vaccinia viruses expressing H-2K^b (*A*) and H-2D^d (*B*). The cells were treated with 100 U γ-IFN for 48 h before vaccinia infection. After pulsing the cells were chased, and class I molecules were immunoprecipitated and treated with endo H as described in the legend to Fig. 2. The following antibodies were used for immunoprecipitation: anti-H-2K^b α1/α2 (20.8.4S) in A and anti-H-2D^d α1/α2 (34.5.8) in (*B*). X-ray films were scanned by densitometer, the percentage of endo H–resistant forms was calculated and is presented graphically in *B*. Endo H^R, endo H resistant; Endo H^S, endo H sensitive.

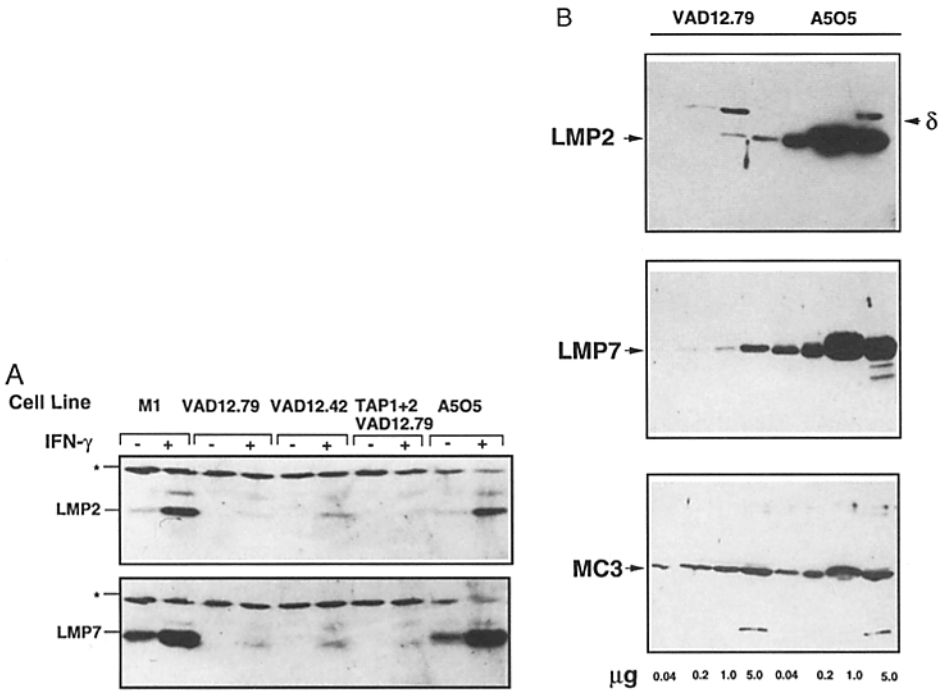

Figure 5. Expression of LMP2 and LMP7 molecules is suppressed in Ad12-transformed cell lines but is induced by γ-IFN. Total cell extracts (*A*) and indicated amounts of purified 20S proteasome extracts from VAD12.79 and A5O5 (*B*) were fractionated by SDS-PAGE and analyzed for the expression of LMP2, LMP7, and MC3 by hybridization of Western blots with affinity purified antisera. Extracts from the following cell lines were analyzed before and after treatment with γ-IFN: M1, A5O5, VAD12.79, VAD12.42, TAP1+TAP2-transfected VAD12.79 (*A*). A cross-reaction with the δ protein is indicated. *Unidentified band.

fore, that proteasomes prepared from VAD12.79 and A5O5 differ in the frequency at which they cleave specific sites in the pp89 polypeptide.

Reexpression of LMP2 and LMP7 in TAP-reconstituted Ad12-transformed Cells Enhances but Does Not Completely Restore the Expression of H-2 Class I Molecules. To determine whether reconstitution of LMP expression could restore transport and cell surface expression of class I genes, a TAP1+TAP2-transfected VAD12.79 cell line was further transfected with plasmid constructs expressing LMP2 and LMP7. Fig. 10 shows the LMP levels in the mixed population and three individual clones that express both LMP2 and LMP7. The clones showed levels of LMP2 and LMP7 which were significantly higher than that of M1 or A505. FACS® analyses of class I cell surface expression of LMP2+ LMP7-expressing clones or of the mixed populations is shown in Fig. 11. The figure shows that LMP expression enhanced cell surface expression of the class I antigens. However, while the expression of PD1 was markedly enhanced such that the mean fluorescence/cell increased by at least twofold, the expression of H-2D^b was increased by 1.2–1.7-fold and was still two- to threefold lower than that of a normal cell line. These results correlated with transport efficiency of these molecules as analyzed by pulse-chase experiments (data not shown).

Discussion

MHC class I molecules are a family of highly polymorphic cell surface glycoproteins whose primary function is to bind and present foreign peptides to CTL (1, 7, 8), thereby controlling pathogenic infections and tumor progression (2–4). Cumulative recent data attest to the fact that suppression of class I cell surface expression involves interference with the transit of these molecules to the cell surface of certain tumor cells (30–33). Several human tumors, such as cervical carcinomas, lung carcinomas, and Burkitt's lymphomas demonstrate direct correlation between reduced levels of class I antigens, escape from CTL recognition, and tumor progression. Murine tumors transformed by adenoviruses have served as excellent experimental model systems for proving that escape from immune recognition in vitro and in vivo is at least in part due to suppression of class I expression (5, 37, 59, 60). In addition, since these tumors are transformed in vitro, the reduced levels of class I antigens do not result from in vivo selection of class I negative cells as probably occurs in human tumors. Thus, the tumor cell lines described in our present study are appropriate models for studying active suppression caused by either viral proteins or cellular proteins induced by viral transformation. Mechanisms involved in regulation of class I molecule expression have been analyzed in parallel in Ad12-transformed, in Ad5-transformed, and in normal cell lines, all originating from the same pool of parental embryonal fibroblasts (31, 55, and the present study). Since Ad5-transformed and normal cell lines exhibit normal levels of class I antigens and are not tumorigenic in vivo, they provide adequate controls for Ad12-transformed cell lines.

In the present study and in our previous report (31) we demonstrated that in Ad12-transformed cell lines, the expression of most genes definitely associated with antigen processing and presentation is differentially reduced. According to the degree of suppression, these genes can be divided into two groups: The first includes TAP2, LMP2, and LMP7, which exhibit a >100-fold reduction in their encoded steady state mRNA levels, with both mRNA and protein products barely detectable. The second group includes TAP1, class I heavy chains, and β2m genes showing

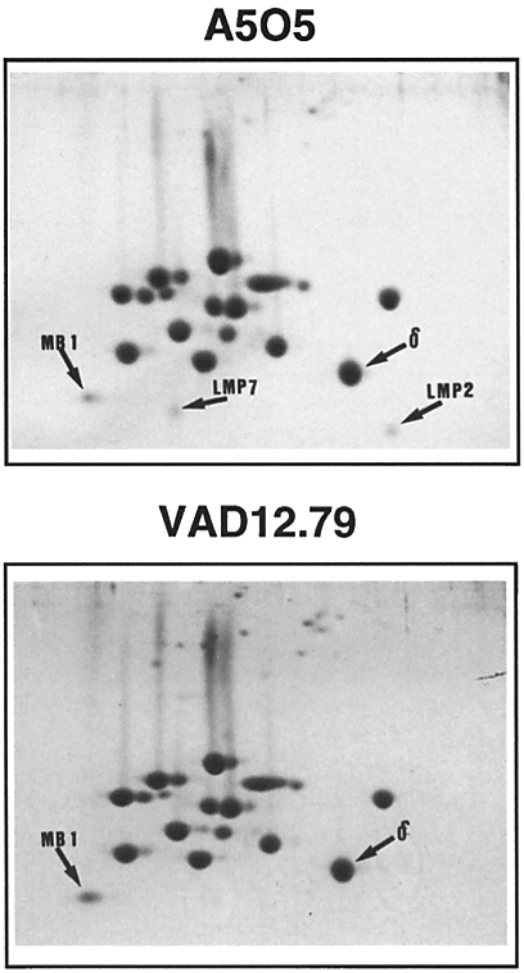

Figure 6. Analysis of 20S proteasome subunits from A5O5 and VAD12.79 cells. Isolated 20S proteasomes from A5O5 and VAD12.79 cells were analyzed by nonequilibrium two-dimensional gel electrophoresis. LMP2, LMP7, MB1, and the δ subunits are marked with arrows.

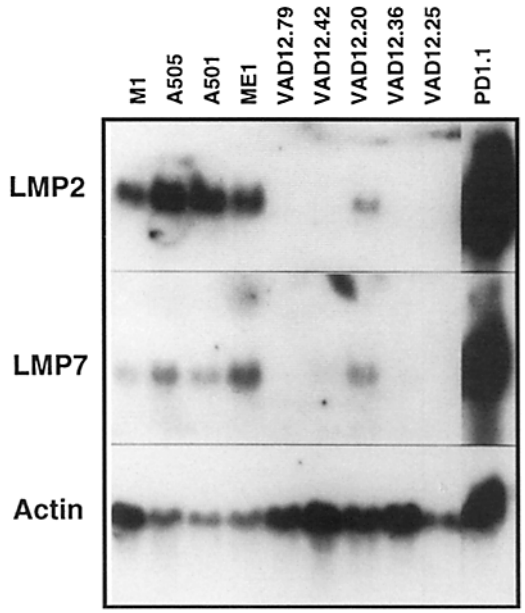

Figure 7. Steady state levels of LMP2 and LMP7 mRNA are reduced in Ad12-transformed cells. Northern analysis of mRNA from normal (*M1*), E1Ad5-transformed (*A5O5*, *A5O1*), Ad5-transformed (*ME1*), Ad12-transformed (*VAD12.79*, *VAD12.42*, *VAD12.20*, *VAD12.36*, *VAD12.25*) cell lines and the RadLV-transformed thymoma (*PD1.1*) was carried out as described in the legend to Fig. 2 *B*. The RNA was hybridized with probes specific for LMP2, LMP7, and Actin as indicated in the figure.

a 2–10-fold reduction in their steady state mRNA levels, with gene expression varying among individual cell lines. The data suggest that there is at least one common regulatory element which controls transcription of these genes, and is affected by viral transformation. Nonetheless, it is clear that the contribution of this yet unidentified regulatory element to the transcription efficiency of the different genes varies, resulting either in complete or partial reduction in their transcription. The E1A-encoded proteins are involved in interactions among various nuclear proteins, and can either enhance or suppress gene transcription (61). Since adenovirus-transformed cells express high levels of these proteins (data not shown), it is conceivable that they are involved in the downregulation of genes associated with antigen processing and presentation. The possibility that multiple suppressive mechanisms affect the transcription of the various genes via different regulatory elements cannot be ruled out. However, it seems less likely in view of the fact that all the genes described above are destined to function in antigen processing and presentation and it is more conceivable that they are regulated by common elements. In addition, the class I heavy chain, TAP and LMP genes are located in the MHC locus, and therefore could have evolved from a common ancestral gene and maintain common associated sequences.

Ad12-transformed cells are the first murine tumor system described where both peptide transporter and LMP genes are downregulated as a result of a transformation event. Restifo et al. (30) previously identified human cancers with defective antigen processing, which do not express TAP and LMP mRNA. However, the investigators did not determine whether this deficiency was 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. We now show that both sets of genes are expressed in the parental fibroblasts and in Ad5-transformed cells that originated from the same pool of embryonal fibroblasts, thereby establishing that suppression of these genes is clearly associated with the transformation event. Moreover, we have confirmed that expression of TAP genes is essential to the expression of class I antigens in this system (31, and Fig. 1 *A*). The latter conclusion is in agreement with data obtained in TAP-negative mutant cell lines such as RMA/S and LBL 721.174, where transfection of TAP genes reconstitutes class I expression and antigen presentation (7, 8, 12, 13). However, we now find that reexpression of TAP genes is essential but not sufficient for restoration of maximal class I molecule expression. In TAP-transfected Ad12-transformed cells, cell surface expression of the transgene product PD1

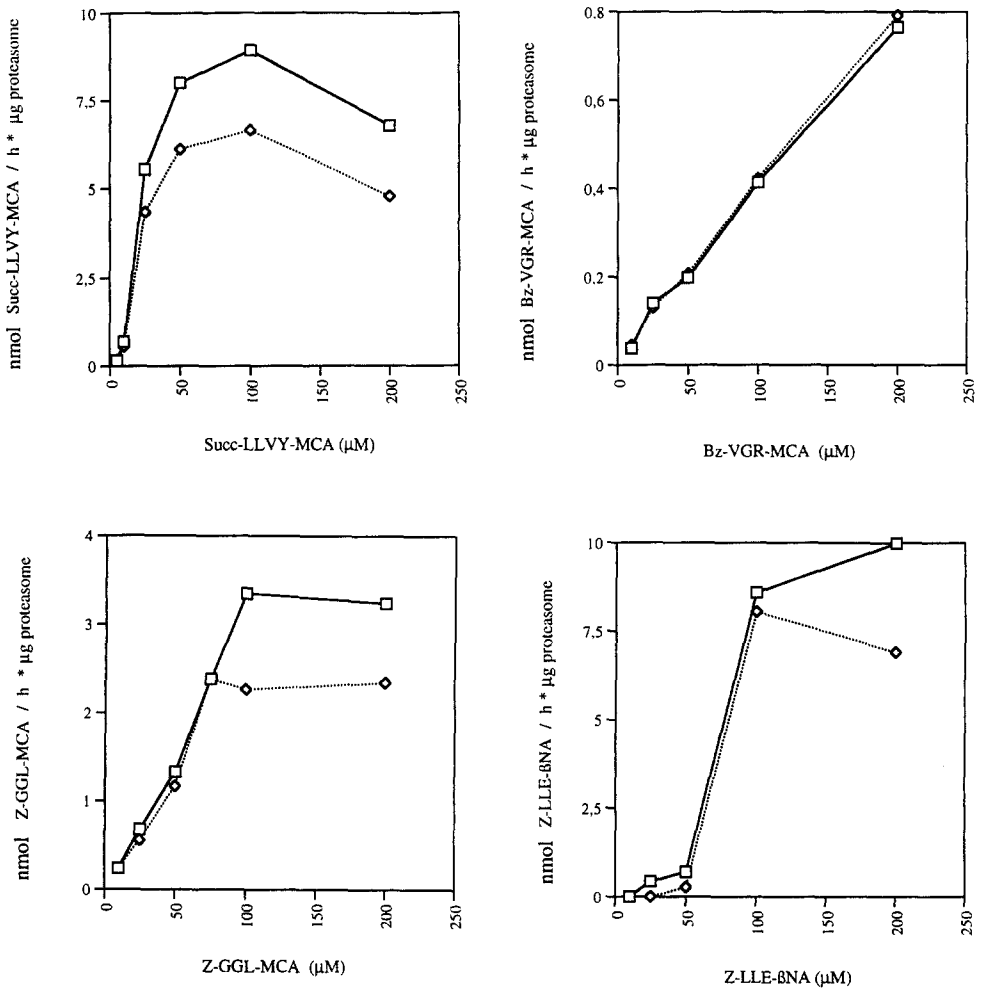

Figure 8. Peptide hydrolyzing activities of 20S proteasomes from A5O5 and VAD12.79 cells. Hydrolyzing activities of 10 µg 20S proteasomes isolated from VAD12.79 (□) and A5O5 (◇) were assayed by using fluorogenic substrates at a range of 0–250 µM. The following substrates were used: Succ-LLVY-MCA (chymotrypsin-like) Z-GGL-MCA (chymotrypsin-like); Bz-VGR-MCA (trypsin-like); d)Z-LLE-βNA (PGPH).

was completely restored, but the expression of H-2D^b was only partially reconstituted (Fig. 1 *A*). Moreover, metabolic labeling of these cells followed by chase and immunoprecipitation (Fig. 2) revealed that most H-2D^b molecules did not acquire endo H resistance over a long chase period. The same results were obtained with cells infected with recombinant vaccinia viruses expressing human TAP1 and TAP2 molecules (Fig. 3 *B*). Since the assembly and transport of a stable class I complex is dependent upon each of its components (49, 57), we tested whether limiting amounts of either β2m or class I heavy chains would interfere with these processes in TAP-transfected or VAC-TAP–infected Ad12-transformed cells. Infection with a recombinant vaccinia virus expressing human β2m, or the addition of recombinant β2m to cell extracts, did not enhance assembly or transport of H-2D^b (data not shown). Infection with vaccinia viruses expressing H-2D^d and H-2K^b (Figs. 3 *A* and 4 *A*) revealed that fully conformed molecules were transported to the cell surface as efficiently as in E1Ad5-transformed cells. However, similarly to the results obtained for H-2D^b, only a small fraction (10–20%) of H-2D^d molecules acquired a conformation allowing efficient transport to the cell surface. This implies the necessity for additional factor(s). Treatment of TAP-transfected Ad12-transformed cells with γ-IFN before infection with the recombinant vaccinia virus resulted in enhanced transport of H-2D^d and H-2K^b (Fig. 4). These data can be explained as follows: First, since the third component of the class I complex is a peptide, it is plausible that the amount of selective sets of peptides (with high binding affinity to H-2 molecules) is limiting in TAP-transfected cells, despite the overexpression of peptide transporter genes. Second, the rate of transport of different class I molecules through cell organelles depends on their conformation, and is determined by polymorphic amino acid residues in the α1/α2 domain (62–65). A specific differential effect on transport of human class I molecules was recently demonstrated in HSV-infected cells (66). It is likely that class I molecules, by acquiring various conformations during biosynthesis and assembly, display a range of affinities in their association to chaperons such as calnexin and BiP (67, 68). It is conceivable that alterations in the amount, affinity to class I heavy chains, or function of such chaperons in Ad12-transformed cells could differentially affect the transport of these molecules. It bears mention that one of the chaperons (gp96), which binds peptides and may be involved in antigen presentation (69), is induced by γ-IFN, a treatment that facilitated the transport of H-2 molecules in our experiments.

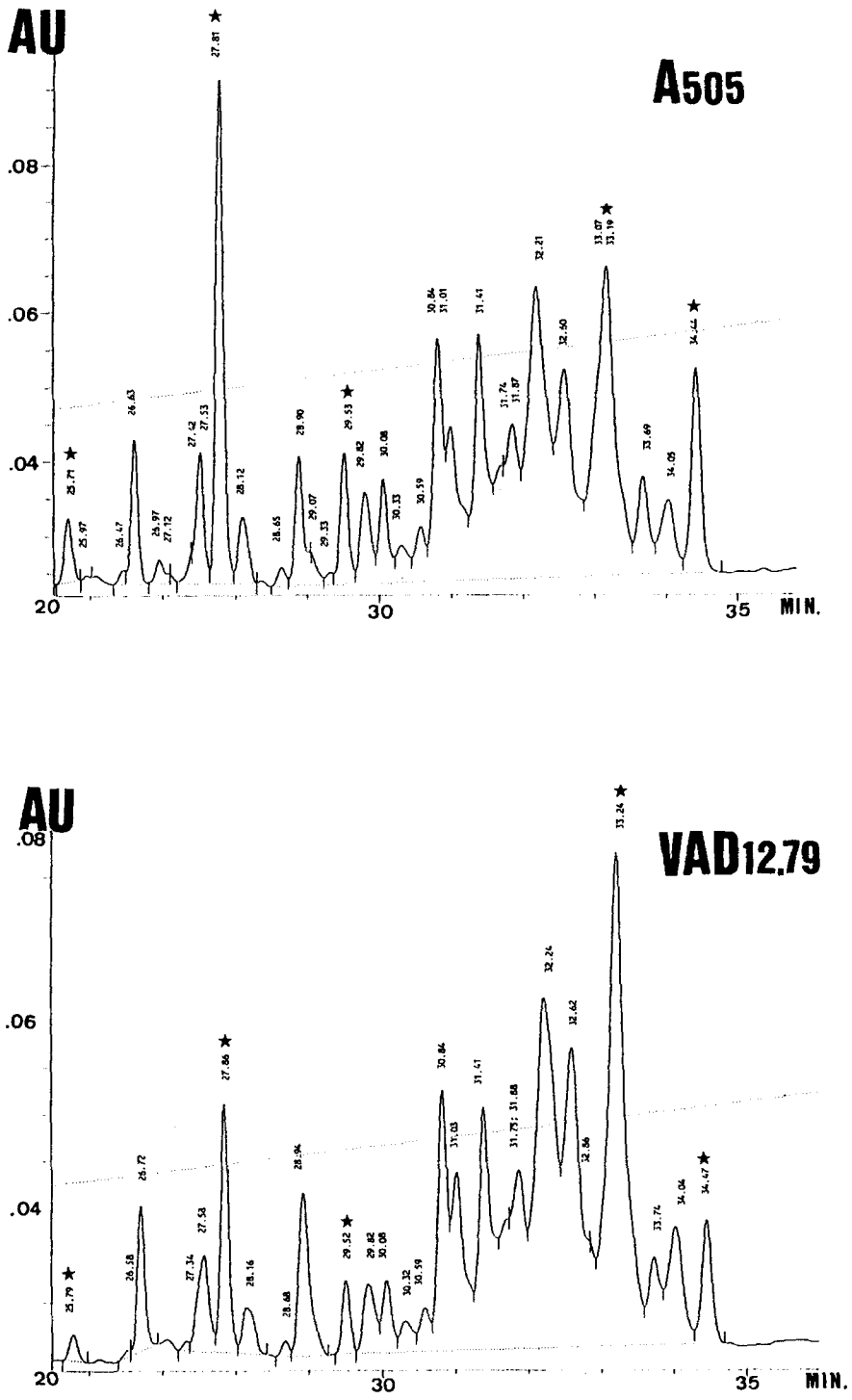

Figure 9. Quantitative differences in cleavage products of pp89 25-mer polypeptide by 20S proteasomes prepared from VAD12.79 and A5O5 cells. The pp89 25-mer substrate was incubated in the presence of 20S proteasomes from A5O5 or VAD12.79 cells. After complete turnover of the substrate, the peptide mixture was separated by reverse phase HPLC. The peptide region (retention time 20–35 min) of original chromatographs are shown. Peaks exhibiting an over twofold difference between the cell lines are marked with a star. AU, absorbance units.

Our data demonstrate a direct correlation between the inhibition in transport of H-2 molecules and the total suppressed expression of LMP2 and LMP7. Ad12-transformed cells were totally devoid of LMP2 and LMP7 molecules (Figs. 5–7), resulting in quantitative alterations in proteolytic activities mediated by the 20S proteasomes preparations from these cells (Figs. 8 and 9). γ-IFN, which induces LMP expression, also induced the efficient transport of the H-2 molecules expressed by a recombinant vaccinia viruses in TAP-transfected cells. Since both TAP and H-2 heavy chains were overproduced in these cells, it is feasible that the induction of LMPs or another unidentified factor which is interferon regulated resulted in enhanced transport of H-2 molecules. It should be emphasized that the inhibition of H-2D^b transport in TAP-transfected Ad12-transformed cells is comparable with the selective and inefficient antigen presentation by H-2D^b molecules reported by Van Kaer et al. in LMP2 mutant mice (70) and by Cerundolo et

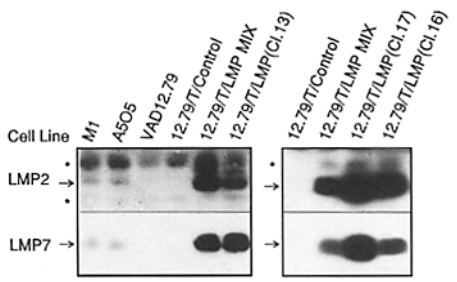

Figure 10. Expression of LMP2 and LMP7 in LMP-transfected cells. Western analysis of cell extracts from LMP-transfected TAP-reconstituted VAD12.79 (*12.79/T/LMP*) (mixed transfectants) or individual clones, and control cell lines (M1, A5O5, TAP-reconstituted puromycin-transfected VAD12.79 [*12.79/T/Control*]) was carried out using 100 μg of protein (as determined by Bradford assay) and antibodies against LMP2 and LMP7 as described in the legend to Fig. 5 *A*. Cl, individual clone.

al. in LBL 721.174 cells transfected with peptide transporter genes (29).

There are several indications that LMP2 and LMP7 play a role in antigen processing and regulate proteasome-mediated proteolytic activities (23–27). However, the observations that in human cell lines lacking LMPs but with peptide transporters reconstituted by transfection, normal cell surface levels of class I MHC are expressed, and these cells are able to present most antigens (71–73), suggest that the absence of LMP2 and LMP7 do not limit the supply of peptides for antigen presentation by class I molecules, at least not in these cells. Nevertheless, several recent reports imply that the expression of LMPs is essential to efficient presentation of selective sets of peptides (28, 29, 70) and for maximal expression of class I antigens (28). Most studies minimizing the role of LMPs for class I expression either used cells of lymphoid origin (71–73) or tested cell surface expression of class I antigens and antigen presentation by T

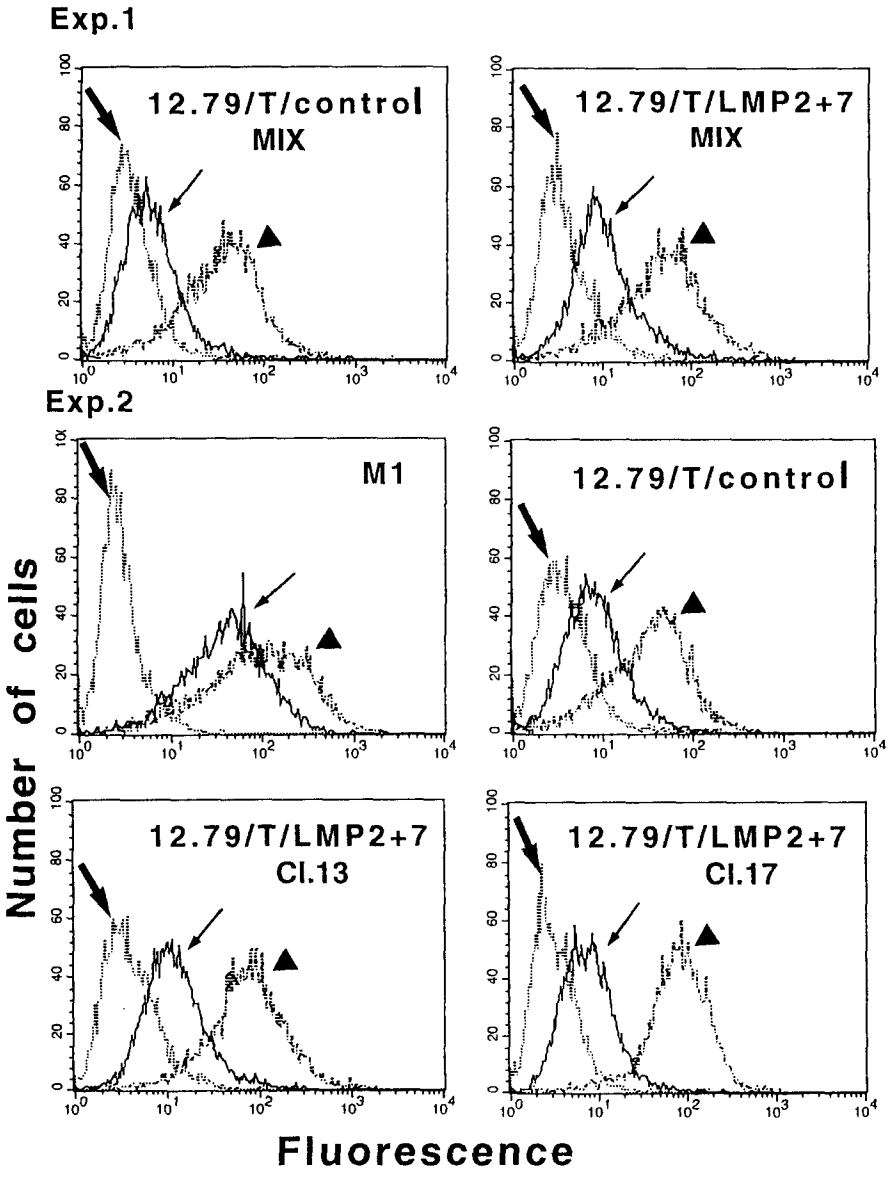

Figure 11. Expression of class I antigens in LMP-transfected TAP-reconstituted Ad12-transformed cell lines. Cell surface expression of class I antigens on the normal cell line (*M1*), TAP-reconstituted Ad12-transformed cell line transfected with puromycin-resistance gene (*12.79/T/control*), or cotransfected with LMP2+LMP7 (mixed transfectants, *MIX*; individual clones, *C1.13*, *C1.17*) were analyzed by immunofluorescence staining with the following antibodies: PT85A (anti-PD1); (*arrowhead*), and 27.11.13 (anti-H-2D^b) (*narrow arrow*). Second antibody control (*fat arrow*). Cl, individual clone.

cells originating in LMP knockout mice (28, 71). However, the effects of LMP deficiency on the constitutive expression and transport of class I molecules in other cells have not been analyzed in detail. It is possible that LMP2 and LMP7 play a more critical role in the constitutive expression of class I molecules in fibroblasts and possibly also in other cells not of lymphoid origin. This is entirely likely since lymphoid cells express higher levels of peptide transporter genes (data not shown), and perhaps additional cytosolic proteases that may be used for antigen processing. Moreover, Brown et al. (74) reported that proteasomes are structurally diverse, and can exist in several distinct compositions within mammalian tissues and cell lines and consequently, may carry out diverse proteolytic activities. Differences between lymphocytes and fibroblasts in proteasome composition could result, therefore, in quantitative and qualitative differences in peptide production between the two cell types. In transformed cells where only small amounts of the total peptides are tumor-associated antigens, alterations in cleavage specificities as a result of LMP2/LMP7 downregulation might completely abolish their generation and hence their recognition by the immune system.

To determine whether LMP2 and LMP7 deficiencies could account for the downregulation of expression of class I molecules in Ad12-transformed cells, TAP-reconstituted Ad12-transformed cells were transfected with LMP2 and LMP7 genes. High stable expression of LMP2 and LMP7 were obtained (Fig. 10) that resulted in enhancement of cell surface expression of PD1 and H-2D^b (Fig. 11). Despite the fact that the transfected cell lines expressed at least 10-fold higher levels of TAP and LMP molecules, transport and cell surface expression of H-2D^b did not reach that of normal cell lines or E1Ad5-transformed cell lines. These data, supported by the fact that even in the presence of high levels of class I and TAP molecules full restoration of class I transport could only be achieved by γ-IFN treatment (Fig. 4), suggest the existence of an additional factor that plays a role in stabilization and transport of the class I complex, and that the expression or function of this factor is IFN regulated. The expression of the 29-kD subunit of the γ-IFN–inducible 11S regulator of the 20S proteasome cannot account for the difference in MHC class I expression as it is equally apparent in Northern and Western analyses in the VAD12.79 and A5O5 cell lines (Soza, A., unpublished results).

The cell lines described in this study are so far the only murine cells in which both LMP2 and LMP7 are knocked out as a result of a transformation event. A previously described hepatoma cell line (74) that does not express LMP2 was used to analyze and characterize distinct proteasome subsets. However, it is probable that this cell line represents normal hepatocytes and does not reflect a specific case of LMP2 downregulation resulting from malignant transformation. Adenovirus-transformed cell lines are unique in that patterns of gene regulation can be compared with those of the normal parental cells and as shown in this study, may be used for the identification of novel factors that play a role in class I transport and in antigen processing. Such cell lines are also valuable tools for studying the transcriptional regulation of peptide transporter and LMP genes, and for further analyses of proteasome subunits assembly as well as the class I complex assembly and transport in normal and transformed cells.

Our results indicate that suppression of peptide transporter, LMP genes, and possible additional factors that are essential for class I transport, is caused by viral transformation and may provide escape mechanisms from immune surveillance. Moreover, only certain oncogenic viruses seem to have the capacity to cause such suppression. Transformed cells that do not express peptide transporter and LMP genes and do not transport class I molecules presenting an immunogenic tumor-associated peptide, may undergo selection in vivo and develop into nonimmunogenic tumors with oncogenic potential. Hence, this system provides a novel model for evaluating the roles of multiple factors in the class I biosynthetic pathway during tumor progression.

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