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Evidence of Selective Processing of Immunodominant Epitopes in Virally Infected Cells

Qian-Jin Zhang,* Susan S. Chen,* Carol-Ann Saari,* Maria G. Massucci,‡ Frank Tufaro,† and Wilfred A. Jefferies2*

Recent advances in clarifying the molecular mechanisms involved in Ag processing and presentation have relied heavily on the use of somatic cell mutants deficient in proteasome subunits, TAP transporter, and cell surface expression of MHC class I molecules. Of particular interest currently are those mutants that lack specific protease activity involved in the generation of antigenic peptides. It is theoretically possible that deficiencies of this nature could selectively prevent the cleavage of certain peptide bonds and thus generate only a subset of antigenic peptides. Gro29/K b cell line is derived from the wild-type murine Ltk - cell line. This cell line is one example of a mutant that lacks specific protease activities. This deficiency manifests itself in an inability to generate a subset of immunodominant peptide epitopes derived from vesicular stomatitis virus and herpes simplex virus. This in turn leads to a general inability to present these viral epitopes to cytotoxic T lymphocytes (CTL). These studies describe a unique Ag processing deficiency and provide new insight into the role of proteasome-independent proteases in MHC class I-restricted peptide generation. The Journal of Immunology, 2000, 164: 4513–4521.

The presentation of endogenously derived antigenic peptides to CTL requires degradation of antigenic proteins in the cytosol, transport of the generated short peptides into the lumen of the endoplasmic reticulum (ER) for assembly with MHC class I/β2-microglobulin (β2m), and expression of the trimeric complexes on cell surface (1). Despite the presence of multiple peptide/MHC complexes on the cell surface, T cells selectively respond to specific complexes of immunodominant epitopes to facilitate clearance of the pathogens in vivo (2, 3). Several mechanisms have been described which appear to influence the immunodominance of T cell determinants. Stability and/or affinity of MHC class I-peptide complexes on the cell surface (4–6) influence specific T cell repertoires. The degradation of antigenic proteins by the cytosolic multicatalytic proteasome (7) and by other types of proteases (8–11) is one of the critical elements for selecting and processing peptides. In addition, selecting an immunodominant epitope may require efficient transport of specific peptide into the ER lumen from the cytosol by the TAP heterodimer. This process selects peptides based on their size and sequence characteristics (12, 13). The peptide transport activity of TAP has often been observed in tumor cells and mutant cells to correlate with the levels of TAP expression (14, 15). The T2 cell line, which lacks TAP genes, cannot actively transport peptides into the ER lumen in an ATP-dependent fashion (15). Burkitt’s lymphoma (BL) cell lines exhibit different transport activities proportional to varying levels of TAP expression (14). The TAP selectivity appears to be partially associated with species variants or alleles of the TAP heterodimer (16, 17). However, it is not clear how low levels of TAP expression influence antigenic peptide transport selection and immunodominance in class I-restricted CTL recognition.

In the present study we have analyzed the mechanisms of selective presentation of immunodominant epitopes derived from vesicular stomatitis virus (VSV) and herpes simplex virus (HSV) by using an H-2K b transfectant of the murine mutant Ltk - fibroblast cell line, Gro29/K b. Two antigenic peptides, VSV-Np 221–250 and HSV-gB 498–505, derived from VSV nucleoprotein and HSV glycoprotein B, respectively, are known to be K b-restricted immunodominant epitopes (18, 19). These two epitopes possess strong immunogenicity and antigenicity. The primary CTL response focuses on these two epitopes (19–22) and there is a concomitant high frequency of CTL precursors directed toward these epitopes (23, 24). The original Gro29 cell line was isolated from the surviving cells after mutagenization using ethyl methanesulfonate before HSV-1 infection (25). Analysis of this cell line has revealed several phenotypic defects. First, this cell line is unable to fully assemble and propagate HSV-1 and VSV at late stages of infection (25). Second, intracellular transport of viral glycoproteins (25) and H-2K b molecules (26) is slow. Third, it is unable to proteolytically process the pseudorabies virus envelope gII glycoprotein after viral infection (27). In our previous study of H-2K b-restricted Ag presentation, Gro29 cells were shown to be sensitive to influenza- and allo-specific CTL lysis, and its ability to present HSV Ag was seriously impaired. This suggests that such a selective Ag processing pathway may be dependent on a cellular function that is also required for viral maturation and egress (26). We have analyzed the molecular basis of the defect in Gro29/K b cells and found that the Ag processing deficiency seriously hampered the generation of...
two Kb-restricted immunogenic epitopes and consequently impaired Ag presentation.

**Materials and Methods**

### Mice

C57BL/6 (H-2b) and C3H (H-2k) mouse strains were bred at the Animal Care Centre at the University of British Columbia. All mice used for the experiments were 6–12 wk old and were maintained in accordance with the guidelines of the Canadian Council on Animal Care.

### Cell lines and cell culture

The H-2Kb transfectants of Ltk- and Gro29 cell lines (H-2b heterotype) (25) were originally derived from mouse L cells transfected with a PB322-based H-2Kb expression vector and were designated as Ltk-/Kb and Gro29/Kb. Both cell lines and their parental lines were maintained in conventional DMEM (Life Technologies, Rockville, MD) supplemented with 10% FCS, 2 mM t-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES. The murine CMT 64 cell line, which does not express the TAP1/TAP2 heterodimer (28), was maintained in the same medium. The murine cell lines RMA (H-2b) and BW5147 (H-2b) (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium containing the same supplements described above.

### MHC class I expression

Total MHC class I expression was detected by one-dimensional isoelectric focusing (1D-IEF). Cells were metabolically labeled by culturing for 6 h at 37°C in methionine-free RPMI 1640 containing 100 μCi [35S]methionine and 5% FCS. Labeled cells were then lysed in buffer containing 0.5% (v/v) Nonidet P-40 (NP-40). After preclearing the cell lysates, immunoprecipitations with an H-2Kb exon 8-specific rabbit antisera (29), which recognizes the cytoplasmic tail of both free and β2m-assembled H-2Kb heavy chains, was performed using equal amounts of TCA-precipitable radioactivity. The immunoprecipitates were digested for 3 h at 37°C with 5 μg of chymotrypsin. After washing twice with PBS, the cells were resuspended in 50 μl of NP-40-conjugated secondary Ab and incubated for a further 30 min at 4°C. All samples were then washed twice, and the mean logarithmetic fluorescence intensity was measured by a FACSanalyser (Becton Dickinson, Mountain View, CA).

### Detection of TAP heterodimer expression and activities

The levels of TAP1 and TAP2 expression were determined by immunoblotting. Total extract from 5 × 105 cells treated with or without 150 μM IFN-γ for 2 days were separated on 10% SDS-PAGE and blotted onto polyvinylidene difluoride. The blots were probed with TAP C terminus-specific rabbit antisera (gifts from Dr. J. J. Monaco) at either a 1:10,000 dilution for anti-mouse TAP1 or a 1:6,000 dilution for anti-mouse TAP2. The blots were then incubated with HRP-labeled anti-rabbit Ab at a 1:500 dilution. The immunocomplexes were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham, U.K.) and were quantitatively assessed by a densitometry scan. For immunoblotting loading control, GADPH protein was detected. The mAb (Chemicon International, Temecula, CA) against GADPH was used at 1:600 dilution and HRP-labeled anti-mouse Ab was used at 1:100,000 dilution.

### TAP heterodimer activities were detected by streptolysin-O-mediated peptide transport assays as described by Neefjes et al. (15) with minor modifications. Human TAP2 minigene at 10:1 multiplicity of infection (moi) or at the indicated concentrations. The targets were labeled with Na[35S]CrO4 (100 μCi/105 cells) for 1 h at 37°C, and cytotoxic activity was assayed in a standard 4-h 51Cr-release assay. The cytotoxicity assays were done in triplicate in 96 V-shaped-well plates at 100:1 E:T ratios. For peptide sensitization, one aliquot of the infected targets was pulsed with 10 μM of the synthetic cysteine-reactive viral peptide during the final 5 min of the cytotoxicity assays. Tests of HPLC fractions were performed by adding 20 μl of the peptide preparations to 5 × 103 labeled targets, after which the plates were incubated for 1 h at 37°C before addition of the effectors.
Peptide toxicities were checked in each assay and were always less than 10%.

Results

Expression of H-2K<sub>b</sub> gene in the Ltk<sup>−/K<sub>b</sub></sup> and the Gro29/K<sub>b</sub> cell lines

In previous studies we have shown that the Gro29 mutant cells exhibit selective presentation of viral Ags via H-2K<sub>k</sub> molecules (26). The precise epitopes from herpes simplex and influenza viruses that associate with K<sub>k</sub> molecules are not known (for HSV) or are diverse (for influenza) (33). In addition, we are unable to generate H-2K<sub>k</sub>-restricted CTL specific for the VSV virus. To investigate this phenomenon of selective Ag presentation further, we transfected the H-2K<sub>b</sub> gene into Gro29 cells and its parental Ltk<sup>−/K<sub>b</sub></sup> cells and examined presentation of the K<sub>b</sub>-restricted viral immunodominant epitopes. Expression of H-2K<sub>b</sub> in the transfectants was determined by 1D-IEF with RMA cells used as a control. [35S]-labeled MHC class I polypeptides were immunoprecipitated from cell lysates containing equal amounts of radioactive lysates of metabolically labeled RMA, Ltk<sup>−/K<sub>k</sub></sup>, and Gro29/K<sub>b</sub> cell lines by using a rabbit antiserum recognizing the cytoplasmic tail of both free and β<sub>M</sub>-assembled H-2K<sub>b</sub> heavy chains. Two aliquots of immunoprecipitated material from each sample were treated with or without neuraminidase for 3 h at 37°C. MHC class I heavy and light polypeptides were resolved by 1D-IEF.

Peptide toxicities were checked in each assay and were always less than 10%.

FIGURE 1. H-2K<sub>b</sub> expression detected by 1D-IEF. MHC class I molecules were immunoprecipitated from equal amounts of radioactive lysates of metabolically labeled RMA, Ltk<sup>−/K<sub>k</sub></sup>, and Gro29/K<sub>b</sub> cell lines by using a rabbit antiserum recognizing the cytoplasmic tail of both free and β<sub>M</sub>-assembled H-2K<sub>b</sub> heavy chains. Two aliquots of immunoprecipitated material from each sample were treated with or without neuraminidase for 3 h at 37°C. MHC class I heavy and light polypeptides were resolved by 1D-IEF.

FIGURE 2. Presentation of VSV and HSV Ags by Ltk<sup>−/K<sub>b</sub></sup>, Gro29/K<sub>b</sub>, and RMA cells. The cells were either infected with or without VSV or HSV at 10:1 moi overnight or pulsed with 10<sup>−7</sup> M synthetic peptide during 5<sup>1</sup>Cr-labeling and then were tested for sensitivity to lysis by the CTL at 100:1 ratio of the E:T ratio. A, Cytotoxicity assays were performed with H-2K<sub>b</sub>-restricted CTL against either VSV-Np<sub>52–59</sub> Ag (top panel) or HSV-gB<sub>498–505</sub> Ag (bottom panel). B, Cytotoxicity assays were conducted by using H-2K<sub>k</sub>-restricted CTL against HSV Ags.

Two viral peptides, VSV-Np<sub>52–59</sub> and HSV-gB<sub>498–505</sub>, have been reported to be H-2K<sub>b</sub>-restricted immunodominant epitopes. A standard 5<sup>1</sup>Cr-release assay was conducted to test whether Gro29/K<sub>b</sub> cells were capable of presenting both well-defined epitopes for CTL lysis. Bulk CTL cultures specific for the two viral epitopes were generated by immunizing C57BL/6 mice with either VSV-1 or HSV-1 before in vitro restimulation of the splenocytes with synthetic peptides corresponding to the VSV-Np<sub>52–59</sub> and HSV-gB<sub>498–505</sub> epitopes. The Gro29/K<sub>b</sub> cell line infected with VSV at 10:1 was consistently 2- to 3-fold less sensitive to lysis by VSV-Np-specific bulk CTL culture than VSV-infected Ltk<sup>−/K<sub>b</sub></sup> and RMA cells (Fig. 2A, top panel). Pulsed with synthetic VSV-Np peptide, all cell lines demonstrated comparably high killing, confirming the presence of sufficient amounts of Kb molecules on the surface of the Gro29/K<sub>b</sub> cell line. Untreated control cells and non-transfectants, Ltk<sup>−/K<sub>k</sub></sup> and Gro29 (data not shown), were not lysed. In contrast to VSV-Np-specific killing, the HSV-infected Gro29/K<sub>b</sub> cells were sensitive to HSV-gB-specific CTL lysis at a level comparable to that of the Ltk<sup>−/K<sub>k</sub></sup> cells (Fig. 2A, bottom panel). To study H-2K<sub>b</sub>-restricted presentation, K<sub>k</sub>-restricted HSV-specific
bulk CTL culture generated from the lymph nodes of C3H mice were used as effectors. The killing of HSV-infected Gro29/Kb cells was significantly lower than the killing of Ltk−/Kb cells infected with HSV (Fig. 2B). This is in accordance with our previous results using the parental cell lines (26). We were unable to use peptide-pulsed targets as controls for this assay because the Kb-restricted epitope derived from HSV has not yet been defined.

The binding of peptides to a relevant MHC class I molecule can induce a conformational change in the α1 and α2 domains that results in recognition by conformational-dependent Abs (34–36). The selective presentation of endogenously processed peptides by the Gro29/Kb cells was further characterized by surface immunofluorescence staining of the Kb-Ag. This was analyzed using two different allospecific mAbs recognizing relevant conformational Kb molecules (Fig. 3). The Ltk−/Kb and the Gro29/Kb cells that were stained with the AF6-88-5.3 Ab (thick lines) had similar surface fluorescence intensity. However, the same cells stained with the 142.23 (thin lines) against surface H-2Kb molecules. The detection was performed by FACS assay. The RMA cells were used as a reference.

**TAP expression and activity are partially deficient in Gro29/Kb cells**

The translocation of naturally processed peptides from the cytosol into the ER lumen requires a functional TAP1 and TAP2 heterodimer and is ATP-dependent (37, 38). Two assays were performed for analyzing TAP expression and TAP activity in Gro29/Kb cells. Immunoblots were probed by two antisera specific for either mouse-TAP1 or -TAP2. The results showed that the expression of TAP1 and TAP2 was down-regulated ~5- to 7-fold in the Gro29/Kb line vs the Ltk−/Kb line (Fig. 4). Not surprisingly, this down-regulation in TAP expression is linked to a defect in its peptide translocation activity. A peptide library-based translocation assay revealed that Gro29/Kb cells were strongly impaired in their ability to transport peptides in an ATP- and TAP-dependent fashion (Table I). Interestingly, in the VSV-Np52–59-modified peptide GVGNTFLG-based translocation assay, Gro29/Kb cells were shown to be capable of transporting this peptide in an ATP-dependent fashion. Although a level of the peptide transport by Gro29/Kb cells was lower than that achieved by Ltk−/Kb cells (Table I), this reduced level of activity did not influence the VSV-Np epitope presentation, as confirmed by a cytotoxicity assay using recombinant vaccinia virus carrying the minigene that encodes the sequence of VSV-Np52–59 (VV-Np). As illustrated in 51Cr-release assay for the titration of VV-Np infection experiment (Fig. 5A), no difference in the sensitivity to killing was observed between VV-Np-infected Gro29/Kb and Ltk−/Kb cells, even at a 0.1:1 moi ratio. IFN-γ treatment restored normal level of TAP expression and peptide library-based peptide transport in Gro29/Kb (Fig. 5, C and D). However, this treatment did not restore VSV-Np epitope presentation in VSV-infected Gro29/Kb cells under the condition shown in Fig. 5B. Thus, under our working condition, the phenomenon of selective Ag presentation by Gro29/Kb cells, in the case of VSV-Np, is unlikely to be ascribed to the defect of TAP expression.

In Gro29/Kb cells, expression of proteasome subunit composition is normal, whereas naturally processed preformed epitopes, VSV-Np, and HSV-gC (potential epitope) are defective. Although Gro29/Kb cells were able to efficiently present a preformed VSV-Np epitope generated from a recombinant vaccinia

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**Table I. ATP- and TAP-dependent peptide translocation in murine cell lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ATP-</th>
<th>ATP+</th>
<th>ATP-</th>
<th>ATP+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ltk−/Kb</td>
<td>2.828 ± 113</td>
<td>10.126 ± 819</td>
<td>3.017 ± 563</td>
<td>11.056 ± 795</td>
</tr>
<tr>
<td>Gro29/Kb</td>
<td>2.728 ± 428</td>
<td>4.826 ± 403</td>
<td>3.129 ± 431</td>
<td>7.209 ± 618</td>
</tr>
</tbody>
</table>

a Transport assays were performed in streptolysin-O-treated cells pulsed with 125I-labeled peptide library.

b Transport assays were performed in streptolysin-O-treated cells pulsed with 125I-labeled VSV-Np-modified peptide GYG-NFTGL.
virus, VSV-infected Gro29/Kb cells present this epitope much less efficiently. A decrease in protease activities may explain this phenomenon. To address this, we first examined proteasome subunit composition in the two cell lines. Two-dimensional gel electrophoresis was performed to separate the 17 different low-molecular-mass polypeptide (LMP) proteasome subunits (39) immunoprecipitated from metabolically labeled Gro29/Kb and Ltk^2/Kb cells. As illustrated in Fig. 6, the MHC-linked, IFN-γ-inducible LMP-2 and LMP-7 subunits and six others were expressed in both cell lines and could be easily identified. When compared, the remaining unidentified subunits between Gro29/Kb and Ltk^2/Kb cell lines, all appeared to be present in both cell lines. The results suggest that proteasome subunit composition in Gro29/Kb is similar or identical with that of Ltk^2/Kb. To investigate other protease activities, the cells were infected with 5:1 VSV or HSV overnight, and the naturally processed peptides were eluted from whole cell lysates and were fractionated by the reverse-HPLC (see Materials and Methods for details). Cytotoxicity assays were then performed using RMA and BW5147 cells as targets pulsed with peptide fractions. As a control, purified synthetic peptide epitopes were prepared using the same procedure to facilitate the identification of the HPLC-eluting position and cytotoxic activity of relevant epitopes. The results of the representative experiments are shown in Fig. 7. Only the 31st HPLC fraction of the naturally processed peptides from VSV-infected Ltk^2/Kb was able to sensitize RMA cells to lysis by VSV-Np-specific CTL (Fig. 7A). This is identical with the elution profile of the synthetic VSV-Np peptide, which also sensitized the RMA target cells (Fig. 7A). In contrast, many HPLC-peptide fractions from VSV-infected Gro29/Kb cells displayed cytotoxic sensitizing activity, whereas the 31st fraction had no activity (Fig. 7A). These results indicate that Ltk^2/Kb cells were able to efficiently generate an immunodominant VSV-Np epitope.
Materials and Methods

The peptide preparation was shown in pulsing targets and were tested for their ability to sensitize targets to effectors were used as the following: the RMA cells pulsed with peptide toxicity assays were performed at 100:1 E:T ratio. The targets and effectivity, the Kβ-restricted and HSV-specific cytotoxic sensitizing activity were assessed. Gro29/Kb cells could generate only different-sized precursors of the VSV-Np epitope. In contrast to VSV peptide sensitizing activity that is independent of the proteasome.

FIGURE 7. Different processing of the viral Ags by Ltk/Kb and Gro29/Kb cells. Naturally processed peptides derived from 10^6 cells of Ltk/Kb and Gro29/Kb infected with 5:1 moi VSV or HSV overnight were pulsed with targets and were tested for their ability to sensitize targets to lysis. The peptide preparation was shown in Materials and Methods. Cytotoxicity assays were performed at 100:1 E:T ratio. The targets and effectors were used as the following: the RMA cells pulsed with peptide fractions from the cells infected with VSV as targets and the H-2Kβ-restricted VSV-specific splenocytes as effectors (A), the RMA cells pulsed with peptide fractions from the cells infected with HSV as targets and the H-2Kβ-restricted HSV-specific splenocytes as effectors (B), and the Bw5147 cells pulsed with peptide fractions from the cells infected with HSV as targets and the H-2Kβ-restricted HSV-specific lymph node cells as effectors (C). ▲ Lysis of the cells pulsed with no peptides; ■ killing for the cells pulsed with the synthetic viral epitopes VSV-Np (A) and HSV-gB (B) that were collected from the peaks of the HPLC fractions at 31st peptide for VSV-Np and 33rd peptide for HSV-gB, respectively; ○ targets loaded with naturally processed peptide fractions separated from HPLC.

whereas Gro29/Kb cells were unable to do so. This suggests that Gro29/Kb cells could generate only different-sized precursors of the VSV-Np epitope. In contrast to VSV peptide sensitizing activity, the Kβ-restricted and HSV-specific cytotoxic sensitizing activity of HPLC fractions from both cell lines infected with HSV were identical (Fig. 7B). The activity occurred in the 33rd fraction (Fig. 7B). This coincided with the elution fraction of the synthetic HSV-gB peptide activity (Fig. 7B). In Fig. 7C, the Kβ-restricted HSV specific killing results are shown. Only the 25th peptide fraction of HSV-infected Ltk/Kb cells contained a sensitizing activity for BW5147 target cells, whereas this activity was observed in HSV-infected Gro29/Kb cells in fraction 30. The different active fractions for both cell lines suggest that the optimal size of Kβ-restricted, HSV-derived epitope, which is likely derived from the VSV-Np has dramatically reduced antigenicity in the Gro29/Kb cell surface. Presentation of the VSV-Np and the HSV-gB epitopes is significantly decreased, whereas the presentation of the HSV-gB epitope is presented normal (Fig. 2). This phenomenon cannot be attributed to a lack of VSV and HSV antigenic protein production during viral infection since it is known that the cells do not block VSV and HSV entry and do not impair the synthesis of viral proteins (25). The expression of all VSV-encoded proteins during the infection is similar in both Gro29 cells and Ltk^- cells (25).

Surface expression of stable MHC class I complexes is thought to be critically important for Ag recognition. Failure to load antigenic peptides onto class I β2m complexes normally results in retention of “empty” complexes in the ER lumen, but their surface expression can be induced by low temperatures (41). In our case, the unsialylated Kβ fraction in the Gro29/Kb cells (Fig. 1) cannot contain “empty” molecules because we could not detect a significant increase in Kβ levels on the surface of Gro29/Kb when the cells were incubated at 26°C overnight (data not shown). Also, the levels of Kβ expression on the Gro29/Kβ cell surface, as shown by two different Abs, are either similar to or exceed the levels on the Ltk^-/Kβ cell surface (Fig. 3), albeit these Abs are conformationally different. In accordance with this observation, our previous study showed that the rate of Kβ surface transport is slower in Gro29 cells compared with the parental cells, Ltk^- whereas the maturation rate of Kβ molecules is similar in both cell lines (26). Thus, the reduced sialylated Kβ molecules in the Gro29/Kb cells are likely due to a retardation of egress from the ER or the cis-Golgi to the plasma membrane.

Little is known about how a reduced rate of MHC class I transport influences its surface expression and thereby its Ag presentation. In this study, one of the immunodominant epitopes, VSV-Np, has dramatically reduced antigenicity in the Gro29/Kb cells (Fig. 2). This phenomenon cannot be attributed to slow transport of MHC class I. Many reports indicate that the immunodominant epitope display a high level of stability on the cell surface complexed with MHC class I heavy chain (5, 6, 42–44) and that the half-lives of such complexes exceed 4 h (6, 42, 44). The immunodominant VSV-Np epitope has recently been described as a “superdominant epitope” (24) because synthetic VSV-Np peptide can compete with three of four other immunodominant peptide epitopes to elicit a strong relevant CTL response in a five-epitope mixture immunization. Thus, it is conceivable that the VSV-Np-bound H-2Kβ complex should be stable and persistent on the cell surface and that its stability should also be more than 4 h. In Gro29 cells, the transport of full 35S-labeled MHC class I molecules to the cell surface occurs within 3 h (26). This suggests that the retardation of MHC class I molecules may not seriously influence the amount of surface Kβ/VSV-Np complex that can trigger CTL response. Thus, we infer that the slow transport of class I molecules in these cells does not play a critical role in selective Ag presentation. More likely, the Gro29/Kb cell line has a defect(s) in the early stages of the Ag presentation pathway.

The complete defect of TAP expression has been reported in many mutated cell lines such as T2 and MHC-S as well as in tumor biopsies (45). Such a defect abolishes ATP-dependent peptide
transport and therefore results in deficient Ag presentation (28, 46). It is noteworthy that in many tumors and tumor cell lines TAP expression is not completely deficient, rather is expression appears to be down-regulated (14, 47, 48). It has been reported that low levels of TAP expression are detected in BL cell lines and are also correlated with impaired TAP function and a defect in Ag presentation. Frisan et al. (14) reported that two EBV-negative and -positive BL cell lines, BL28 and E95B-BL28, with low levels of TAP expression exhibit an impairment of ATP- and TAP-dependent peptide transport. This was observed in a translocation assay performed with a peptide library containing 3240 different peptides. Moreover, Khanna et al. (48) reported a blockage of presentation of an HLA-B8-restricted epitope from EBV nuclear protein 3 in an EBV-negative BL30 cell line that expresses a low level of TAP1 mRNA. The involvement of the transporter was confirmed by the capacity to overcome the defect in cells transfected with a minigene encoding the epitope preceded by an ER-localization signal. However, these observations do not explain the defect in the Gro29/Kb cell line. Lower levels of TAP1 and TAP2 expression were observed in this cell line (Fig. 4). Indeed, an ATP-dependent peptide translocation assay performed with a peptide library, which was identical with that used by Frisan et al. (14), revealed a dramatic reduction of peptide transport in the Gro29/Kb cells (Table I). IFN-γ treatment restored normal levels of TAP expression (Fig. 5C) and recovered its peptide transport function, as measured by a peptide library-based translocation assay (Fig. 5D). These results suggest that TAP function is decreased in Gro29/Kb cells. However, such a defect is not complete because the transport of a VSV-Np-modified peptide is not strongly impaired in this cell line (Table I). Also, this level of reduction does not influence VSV-Np epitope presentation under our working condition (Fig. 5A). Interestingly, IFN-γ treatment does not restore the presentation of VSV-Np epitope by VSV-infected Gro29/Kb cells (Fig. 5B). Thus, selective Ag presentation, at least for VSV-Np epitope, cannot be attributed to the TAP down-regulation event in the Gro29/Kb cells.

It remains uncertain why down-regulation of TAP in Gro29/Kb cells has no significant influence on VSV-Np epitope transport. One explanation is that low levels of TAP expression may preferentially transport a particular set of peptides from the endogenuously processed peptide pool. This may be due to TAP saturation occurring in Gro29/Kb-TAP, and therefore, some selectivity might happen based on affinity of peptides for the transporter or the level of peptides generated in the cytosol.

It has been reported that mouse TAP selectively transports optimal-sized peptides or those slightly longer (49). Although low TAP expression did not significantly influence the VSV-Np epitope transport and presentation that we have observed, the longer precursors of this epitope are likely to influence their transport and presentation. This will be further investigated to understand the significance of these findings for tumor immunity.

The results illustrated in Fig. 7 strongly suggest that the evidence of selective Ag presentation in Gro29/Kb cells is relevant to protease activity. The proteasome, a cytosolic multiple-catalytic complex which contains at least five different activities (50), is believed to play a dominant role in the degradation of endogenous antigenic proteins into MHC class I-restricted peptides (7, 51). Our results suggest that the VSV nucleoprotein is cleaved into many different sizes of VSV-Np epitope precursors in Gro29/Kb cells and strongly implicates the involvement of the proteasome. This is in accordance with the results reported by Dick et al. (52), who found that the purified proteasome is able to process, in vitro, the OVA and β-galactosidase proteins into many different sizes of the epitope precursors. However, we did not find the production of optimal-sized VSV-Np epitope in Gro29/Kb cells as Dick et al. did with the OVA- and β-galactosidase-derived epitopes. The question is whether or not the lack of ability of Gro29/Kb cells to generate the VSV-Np epitope and the Kb-restricted HSV-gC-derived epitope should be attributed to the defects of the proteasome. We have observed proteasome expression by two-dimensional gel electrophoresis. The results revealed that all proteasome subunits expressed in the Gro29/Kb were identical with those observed in parental Ltk-/Kb (Fig. 6), suggesting the existence of the intact proteasome. Thus, it appears that the lack of two epitopes in the Gro29/Kb is not due to a defect of the proteasome subunits. This suggests a lack of other protease activities in the cytosol (53), the ER lumen (9, 10), or the trans-Golgi network (11). The inability to generate optimal-sized VSV-Np and HSV-gC epitopes in Gro29/Kb cells by the proteasome may reflect a difference of the epitopes and/or their flanking sequence, which influence proteasomal cleavage (54). This is supported by the finding that epitope precursors, derived from hemagglutinin glycoprotein, as short as 21 aa required processing by proteasome activities, whereas those 17 aa or shorter did not (55). In Gro29/Kb cells, we still cannot exclude the possibility of a point mutation or mutations having occurred in its proteasome subunits or, alternatively, in the proteasome regulator PA28, which regulates proteasome activity (56).

The evidence that the naturally processed peptide epitope precursors can still sensitize the targets to lysis by relevant CTL suggests that the peptides capable of binding to MHC class I molecules are produced in Gro29/Kb cells. Taking into consideration the peptide length preferences for mouse MHC class I molecules produced in Gro29/Kb cells. Taking into consideration the peptide length preferences for mouse MHC class I (Kb, 8–13 mer; and Dα, 9–15 mer) (57) and the proteasomal degradation limitation of 21 aa of the hemagglutinin-derived epitope-containing translation products (55), we speculate that the size range of the generated epitope precursors for the VSV-Np and the HSV-gC in Gro29/Kb cells are possibly more than 8 and less than 21 aa long. It is not clear yet which other proteases, independent of the proteasome, govern the processing of such precursors. Evidence suggests that protease activities in secretory compartments are required for Ag presentation. Efficient presentation of a 12-mer peptide encoded by a minigene has been shown to require ER-directed expression of a carboxypeptidase (10). An ER-localized amino peptidase has been suggested to preferentially liberate the C-terminal T cell epitope when two different epitopes are linked to each other in tandem (9). Both observations imply that once peptides are translocated into the ER, they are trimmed to optimal size for class I binding by ER-localized proteases. In addition to this, a trans-Golgi network protease, furin, which involves class I-restricted viral Ag processing, has recently been suggested (11). The hepatitis B (HB) virus secretory core protein HBe can efficiently deliver C-terminally located antigenic peptides for presentation in the absence of TAP. This presentation, facilitated by furin, was believed only to require HBe maturation in the Golgi or post-Golgi compartment, processed by furin. In the Gro29/Kb cells, the VSV-Np and the HSV-gC epitopes cannot be generated. This is more likely due to the lack of protease activity or protease activities in the secretory compartment. The reasons are as follows: first, longer precursors of the VSV-Np and the HSV-gC epitopes can be generated (Fig. 7, A and C); and second, the presentation of these precursors occurs on the surface of the Gro29/Kb cells, albeit at very low levels (Fig. 2). This indicates that the longer peptide precursors pass through the class I-restricted Ag presentation pathway without trimming in the secretory compartment. Furthermore, the evidence that Gro29 cells have a defect in trans-Golgi protease...
activity specific for the processing of pseudorabies virus glycoprotein (27) supports this possibility.

In summary, we describe the first evidence of an Ag processing deficiency that is likely linked to a protease activity distinct from the proteasome.

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References


