Comparison of Cell Lines Deficient in Antigen Presentation Reveals a Functional Role for TAP-1 Alone in Antigen Processing

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Summary
Cytotoxic T lymphocytes (CTL) recognize antigenic peptides bound to major histocompatibility complex class I antigens on the cell surface of virus-infected cells. It is believed that the majority of peptides originate from cytoplasmic degradation of proteins assumed to be mediated by the "20S" proteasome. Cytosolic peptides are then translocated, presumably by transporters associated with antigen processing (TAP-1 and -2), into the lumen of the endoplasmic reticulum (ER) where binding and formation of the ternary complex between heavy chain, β2-microglobulin (β2m) and peptide occurs. In this study, we have analyzed and compared the phenotype of two mutant cell lines, the thymoma cell line RMA-S and a small lung carcinoma cell line CMT.64, in order to address the mechanism that underlies the antigen processing deficiency of CMT.64 cells. Unlike RMA-S cells, vesicular stomatitis virus (VSV)-infected CMT.64 cells are not recognized by specific CTL. Interferon γ (IFN-γ) treatment of CMT.64 cells restores the ability of these cells to process and present VSV in the context of K b. We show that although CMT.64 cells express a low level of β2m, the recognition by VSV-specific CTL is not restored by increasing the amount of β2m synthesized in CMT.64 cells. In addition, we find that CMT.64 cells express moderate levels of K b heavy chain molecules, but most of it is unstable and rapidly degraded in the absence of IFN-γ treatment. We infer that the antigen processing deficiency does not lie at the level of β2m or K b production. We also show that the mRNAs for both TAP-1 and -2 are present in RMA and RMA-S cells but are absent in uninduced CMT.64 cells. Upon IFN-γ induction, both mRNAs are highly expressed in CMT.64 cells. In addition, we find that the low molecular mass polypeptides 2 and 7, and additional components of the proteasome are induced by IFN-γ in CMT.64 cells. Finally, introduction of the rat TAP-1 gene in CMT.64 cells restores CTL recognition of VSV-infected cells. These results indicate that a TAP-1 homodimer may translocate peptides in the ER and explain partially the CMT.64 defect and the RMA-S phenotype. These findings link a dysfunction in the transport and/or generation of antigenic peptides to the capacity of tumor cells to evade immunosurveillance and provide a unique model system to dissect this phenomenon.

MHC class I molecules are normally expressed at the cell surface as ternary complexes formed by a heavy chain of 46 kD, a light chain called β2-microglobulin (β2m)1 of 12 kD, and a peptide composed of 8–10 amino acids (1–4). Formation of the ternary complex is thought to involve transport into the lumen of the endoplasmic reticulum (ER) of peptides generated by protein degradation in the cytoplasm (5–7). The study of mutant cell lines selected for their low expression of MHC class I molecules at the cell surface has provided insights into the molecular events required for antigen processing. These studies have allowed the identification of two genes located in the MHC region which encode proteins of the ATP binding cassette (ABC) family. These genes, called transporters associated with antigen processing 1 and

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1 Abbreviations used in this paper: β2m, β2-microglobulin; ER, endoplasmic reticulum; LMP, low molecular mass polypeptide; MOI, multiplicity of infection; N, nucleocapsid; TAP, transporter associated with antigen processing; VSV, vesicular stomatitis virus.

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2 (TAP-1 and -2) have been implicated in transport of peptides from the cytoplasm to the lumen of the ER (8–17). Two other MHC linked genes, low molecular mass polypeptides 2 and 7 (LMP-2 and -7) (18), are components of the proteasome, a cytoplasmic multicatalytic protease complex, that is likely responsible for some aspects of protein degradation for antigen processing (19–25).

The mouse mutant lymphoma cell line RMA-S expresses low levels of class I molecules at the cell surface compared to the wild-type RMA cells (26, 27). Influenza virus–infected RMA-S cells present influenza peptides in the context of Db molecules inefficiently and are only weakly recognized by specific CTL (27). Transfection with the putative transporter gene, TAP-2, complements this deficiency (28, 29). The endogenous TAP-2 gene of RMA-S cells was shown to contain a point mutation that introduces a stop translation codon resulting in an incomplete and defective TAP-2 protein (30). Despite the defective TAP-2 protein in RMA-S cells, antigenic peptides from vesicular stomatitis virus (VSV) bypass the defect and are presented to specific CTL by Kb molecules in RMA-S cells (31, 32). The VSV-nucleocapsid (N) peptide, VSV-N 52-59, was shown earlier to be the major peptide presented by Kb molecules on VSV-infected cells (1). The presence of the wild-type TAP-1 protein in RMA-S cells may be sufficient for translocation of the VSV-N 52-59 peptide to the ER lumen (28–30). Alternatively, the VSV-N 52-59 peptide may not need a functional transporter for transport into the lumen of the ER. Expression of minigene-encoded viral peptide epitopes in T2 cells (33) and in vitro translation and translocation using microsomes from T2 cells (34) support this contention.

A separate class of antigen-processing variants are those in which the assembly and the surface expression of MHC class I molecules are entirely inducible by IFN-γ (35). For example, in the small lung carcinoma cell line CMT.64, recognition by influenza virus–specific CTL does not take place unless induced with IFN-γ (36). The very low amount of all proteasome components present in uninduced CMT.64 cells is presumed to be responsible for their phenotype (19). Exogenous influenza peptides can bind to Db molecules on CMT.64 cells and complement recognition by influenza–specific CTL (36). In addition, we have found that β2m and the VSV-N 52-59 peptides added exogenously to these cells complement recognition by VSV-specific CTL restricted to Kb (37). The amount of β2m and of heavy chains synthesized in these cells may limit the amount of MHC class I expression on the cell surface (37). A dysfunction of the putative peptide transporters and/or in the generation of the peptide may be responsible for the CMT.64 phenotype which may represent a mechanism to downregulate MHC class I expression, a feature common to many carcinomas.

RMA, RMA-S, and CMT.64 cells originate from the same strain of mice, C57Bl/6, and therefore should have the same transporter genes with identical properties regarding the peptide delivery to Db and Kb molecules unless mutated or downregulated (26, 35). This therefore gives us the opportunity to examine the constituents of antigen processing in a controlled system. In this paper, we show that RMA-S and CMT.64 cells have a different phenotype regarding VSV antigen processing and presentation with Kb molecules. We have followed the expression of heavy and light chains, transporters (TAP-1 and -2), and proteasome components (LMP-2 and -7) in RMA, RMA-S, and CMT.64 IFN-γ induced or uninduced cells and we have compared the phenotype of RMA-S and CMT.64 cells regarding antigen presentation in the context of Kb molecules. These data revise the interpretation of the molecular basis of the antigen-processing deficiency in the carcinoma cells CMT.64. In addition, we have reintroduced several components of the antigen-processing machinery in order to examine their ability to complement the CMT.64 phenotype.

Materials and Methods

Animals and Viruses. C57Bl/6 mice were bred at the University of British Columbia breeding facility. Mice were 6–12 wk old and were maintained in accordance with the guidelines of the Canadian Council on Animal Care. VSV was grown on Vero cell monolayers. Vaccinia and A human β2m (hβ2m) vaccinia recombinant were generous gifts from Dr. J. Yewdell (National Institutes of Health, Bethesda, MD).

Cell Lines and Antibodies. CMT.64 cells (H-2d), graciously provided by Dr. L. M. Franks, were in culture supernatant from 28-11-5s and 28-14-8s cells for 45 min with a representative clone are reported (see Fig. 9). As negative controls, clones obtained from a vector DNA transfection were analyzed by Northern blotting. The results obtained with a representative clone are reported (see Fig. 9). As negative controls, clones obtained from a vector DNA transfection were analyzed by Northern blotting. The results obtained with a representative clone are reported (see Fig. 9).

Flow Cytometry Analysis. To determine the cell surface expression of MHC class I molecules, we used FACS® analysis (Becton Dickinson & Co., Mountain View, CA). RMA, RMA-S, and CMT.64 cells were treated with or without recombinant murine IFN-γ at 150–300 U/ml (Genzyme Cytokine Research Products, Cambridge, MA) for 48 h. The cells were collected and incubated overnight in medium without FCS, with VSV-N 52-59 peptide (50 μM) and/or hβ2m (2.5 μg). Peptides were purchased from the University of Victoria, Peptide Synthesis Facility (Victoria, BC, Canada). The cells were then fixed in paraformaldehyde (1.5% in PBS) and analyzed on a FACSscan® cell sorter using the FACSscan® program (Becton Dickinson & Co.).

Values reported in Table 1 are in linear terms representing the average of 5,000 cells. The corrected value (minus the value without first antibodies) is reported.
**Cell Labeling, Pulse-Chase Experiments, Immunoprecipitation, Isoelectric Focusing, and SDS-PAGE.** Cells were washed in MEM medium without methionine 1 h before labeling and labeled with 150 μCi/ml of [35S]methionine for 1 h or as indicated. For the pulse–chase experiments, cells were labeled for 15 min and then chased with normal medium containing an excess of cold methionine. Labeled cells were solubilized with 1 ml of 20 mM Tris-HCl, pH 7.6, containing 0.12 M NaCl, 4 mM MgCl₂, and 1% NP-40; PMSF (a protease inhibitor) was added to a final concentration of 20 μg/ml before use. After 15 min on ice, particulate material was removed by centrifugation. The supernatant was used for immunoprecipitation of labeled antigens. Labeled solubilized antigens were first precleared with 2 μl of normal rabbit serum for 45 min at 4°C followed by 50 μl of protein A-Sepharose (1:1 in solubilization buffer) for another 45 min at 4°C. Protein A-Sepharose was removed by a quick centrifugation. The precleared supernatant was reacted with the appropriate antibody or immune serum for 1 h at 4°C. 35 μl of protein A-Sepharose was added and incubation continued for a further 30 min. After centrifugation, the beads were washed twice with 0.2% NP-40 in 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 2 mM EDTA, once with 0.2% NP-40 in 10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 2 mM EDTA, and finally with 10 mM Tris-HCl, pH 7.5. One-dimensional isoelectric focusing was performed as previously described (41). SDS-PAGE was carried out as described (42).

**CTL Response Against VSV-infected, IFN-γ-induced Cells.** RMA, RMA-S, and CMT.64 cells were treated with or without IFN-γ at 200 U/ml for 48 h. They were subsequently washed three times with PBS and treated with VSV at a multiplicity of infection (MOI) of 5 in 0.5 ml of medium for 1 h. The cultures were then incubated in a total of 3 ml of growth medium for an additional 4–8 h (as indicated), to allow infection to proceed. Single cell suspensions were treated with 100 μCi 51Cr per 10⁶ cells for 2 h in RPMI 1640 supplemented with 1-glutamine and penicillin/streptomycin in the absence of fetal bovine serum (FBS) and sodium bicarbonate. Alternatively, CMT.64 cells were infected with vaccinia (V), and/or vaccinia-hβ₂m (Vb2) at an MOI of 5 for 5 h followed by superinfection with VSV (MOI, 5) for an additional 4 h. The cells were washed 3 x and subsequently incubated at 10⁶ cells per well in 96-well plates with the effector population at ratios of 100:12.5. Mock-infected cells were used as negative controls. The effector CTL population was generated by immunizing C57Bl/6 mice with VSV at 5 × 10⁶–1 × 10⁶ tissue culture infection dose 50% in the foot pads and ears. On day 5 after immunization, the draining lymph nodes (retropharyngeal and popliteal) were harvested and cultures initiated at 4 × 10⁵ cells/ml in a total volume of 5 ml in 6-well plates. The culture medium consisted of RPMI-1640 supplemented with 5 × 10⁻³ M 2-ME, 10% heat-inactivated FBS, sodium pyruvate, penicillin, streptomycin, 1-glutamine, Hepes, sodium bicarbonate, and 50% NCTC-109. Cultures were incubated for 3 d at 37°C and 5% CO₂ in the absence of exogenous stimulation. The 51Cr-release was measured by a compugamma counter (model 1282 CS; LKB Instruments, Gaithersburg, MD) and the specific 51Cr-release calculated as [(experimental − media control)/(total − media control)] × 100%. The spontaneous release never exceeded 17% of the maximum release.

**RNA Extraction and Northern Analysis.** Total cellular RNA was prepared from cell lines using guanidinium isothiocyanate (GITC). Briefly, the cells were lysed in 4 M GITC then centrifuged (130,000 g for 16 h at 23°C) through a cushion of cesium chloride. After ethanol precipitation, the purified RNA was resuspended in diethyl pyrocarbonate-treated H₂O. 10 μg of each sample was loaded and separated on a 1% agarose gel containing 2.2 M formaldehyde. The gel was blotted onto Hybond N (Amerham Corp., Arlington Heights, IL) and U/V fixed before hybridization. The 32P-labeled probes used for hybridization were as follows: MTP1 and MTP2 (TAP-1 and -2 respectively, kindly provided by Dr. Geoff Butcher), prepared by random priming, and an oligonucleotide specific for β-actin labeled by terminal transference. Hybridization was carried out at 42°C in buffer containing 0.4 M Na₂HPO₄, 50% formalamide, and 7% SDS. Several washes were performed at 42°C under conditions of increasing stringency and the filter exposed to X-OMAT AR film (Eastman Kodak) overnight.

**Results and Discussion**

The small lung carcinoma cell line, CMT.64, was shown to express and assemble MHC class I molecules on the cell surface after IFN-γ treatment (35–37). To understand the molecular deficiency in antigen processing of CMT.64 cells, the
Table 1. Peptides (p), h$\beta_{2m}$ (hbm), and IFN-\(\gamma\) (IFN) Treatment Modifies the Conformation of \(K^b\) and \(D^b\) Expressed on the Cell Surface of RMA, RMA-S, and CMT.64 Cells

| Cell lines | Treatment | Antibodies |
|------------|-----------|-------------|
|            | IFN | p | h$\beta_{2m}$ | K$^b$ spec. | D$^b$ spec. | h$\beta_{2m}$ spec. |
| CMT 64     | -   | - | -             | 5*      | 3           | ND             |
| RMA        | +   | - | +             | 8       | 5           | ND             |
| RMA-S      | +   | - | -             | 26      | 58          | ND             |
| RMA        | +   | - | +             | 35      | 53          | ND             |

Experiment 2

| Cell lines | Treatment | Antibodies |
|------------|-----------|-------------|
| RMA        | +   | - | -             | 346     | 430         | 6              |
|            | +   | + | -             | 606     | 449         | 4              |
| RMA-S      | +   | - | -             | 12      | 4           | 14             |
|            | +   | + | -             | 10      | 1           | 14             |
| RMA-S      | +   | - | +             | 10      | 2           | 76             |
| RMA-S      | +   | + | +             | 41      | 2           | 262            |

Experiment 3

| Cell lines | Treatment | Antibodies |
|------------|-----------|-------------|
| RMA        | -   | - | -             | 164     | 173         | 1              |
| RMA-S      | -   | + | +             | 242     | 182         | 274            |
| RMA-S      | -   | - | -             | 12      | 2           | 1              |
| RMA-S      | -   | + | +             | 70      | 2           | 86             |

* Arbitrary fluorescent units.

**Figure 2.** Amount of h$\beta_{2m}$ synthesized in RMA, RMA-S, and CMT.64 IFN-\(\gamma\) induced or uninduced cells. Cells were labeled for 2 h with \[^{35}S\]methionine, lysed, immunoprecipitated with a rabbit anti-h$\beta_{2m}$ serum, and analyzed by SDS-PAGE. Radioactive proteins are detected after 6-h exposure to a XAR film. CMT.64 cells treated (+) or not (-) with IFN-\(\gamma\), RMA and RMA-S cells were used. (Left) The migration of the molecular weight marker.

Contrasting phenotypes of CMT.64 cells versus RMA-S cells were analyzed. VSV-infected RMA-S cells are recognized as efficiently as the wild-type RMA cells with or without IFN-\(\gamma\) treatment (Fig. 1A), in comparison to VSV-infected CMT.64 cells which are not recognized by specific CTL unless induced by IFN-\(\gamma\) (Fig. 1B). It should be noted that RMA-S, RMA, and CMT.64 cells are equally permissive to infection with VSV as indicated by the number of infective particles produced after infection measured by a TCID$_{50}$ assay (data not shown). We conclude that uninduced CMT.64 cells have a different or additional deficiency to the functionally defective peptide transporter TAP-2 present in RMA-S cells.

Previous experiments have demonstrated that treatment of mutant cells with exogenous peptides and/or human h$\beta_{2m}$ can stabilize "empty" class I molecules at the cell surface (27, 43, 44). RMA, RMA-S, and CMT.64 cells uninduced or induced with IFN-\(\gamma\) were treated overnight with exogenous peptides VSV-N 52-59 at 50 \(\mu\)M in the presence or absence of human h$\beta_{2m}$. VSV-N 52-59 peptides and h$\beta_{2m}$ synergistically increase the expression of the K$^b$ conformational specific epitope recognized by 142.23.3 mAb (Table 1) on RMA and RMA-S cells. VSV-N 52-59 peptides specifically affect the stability and the conformation of the K$^b$ molecules and have no effect on D$^b$ molecules. Human h$\beta_{2m}$ binds to K$^b$ and D$^b$ molecules, which are detected by BBM.1 (anti-human h$\beta_{2m}$ mAb), and appears to stabilize heavy chains before they
can disassemble at the cell surface. A stabilizing effect is not seen after CMT.64 treatment with peptides or β2m alone. Additional treatment of CMT.64 cells with IFN-γ is required for high expression of Kb and Db conformation-specific epitopes on the cell surface (Table 1). We conclude that CMT.64 cells express much lower amounts of empty class I molecules at the cell surface than RMA-S cells.

Earlier work (45) has shown that the presence of β2m and peptides within the lumen of the ER is necessary for efficient assembly and cell surface expression of MHC class I molecules. Fig. 2 shows that CMT.64 cells express a low amount of endogenous β2m (Fig. 2, lane 1). IFN-γ-induced CMT.64 cells express a much higher amount of β2m, which is comparable to the level expressed in RMA and RMA-S cells (Fig. 2, lanes 2-4). To investigate the effect of β2m in CMT.64 cells, we have used a recombinant vaccinia virus to increase the amount of endogenous β2m (Fig. 3, inset). Elevating the amount of β2m synthesized using a recombinant vaccinia virus does not restore CTL recognition of VSV-infected CMT.64 cells (Fig. 3). We conclude that increasing expression of β2m does not induce presentation of VSV-N peptides in the context of Kb molecules. The CMT.64 antigen-processing phenotype is not caused by the low amount of endogenous β2m.

Our previous paper (37) has shown that MHC class I heavy chains are synthesized in significant amounts in CMT.64 cells, but very few molecules are reaching the cell surface unless these cells are treated with IFN-γ. Here the transport of Kb and Db molecules was examined after a pulse-chase labeling of CMT.64, RMA and RMA-S cells and SDS-PAGE analysis of the immunoprecipitated material (for Kb, 142.23.3 mAb was used and for Db, 28-14-8s, an α3-specific mAb was used) (Fig. 4). Despite a similar amount of Kb molecules synthesized in RMA and RMA-S cells (Fig. 4, 0-h chase time), only low amounts of Kb are processed to a higher molecular weight form, indicative of the level of transport which accounts for the surface expression of Kb in RMA-S cells. The processed form is resistant to endoglycosidase H digestion (data not shown) indicating transport out of the ER. The observation that much more β2m is immunoprecipitated with Kb molecules than with Db molecules in RMA-S cells (Fig. 4) may indicate that the mAb 142.23.3 only recognizes the assembled form, heavy and light chains of Kb molecules, whereas the mAb 28-14-8s recognizes the α3 region of Db molecules. The presence of a functional TAP-1 protein in RMA-S cells (30) may be sufficient to enable some peptides to cross the ER membrane and bind a small number of Kb molecules allowing them to go to the cell surface. Also, peptides with lower affinity for Kb molecules may bind and aid the molecules to assemble and go to the cell surface where they dissociate. Much fewer mature processed Db molecules are detected in RMA-S cells after 4-h chase (Fig. 4). This may indicate a lower affinity of Db for β2m and/or fewer peptides available for Db binding. In RMA cells, Kb molecules are processed within 1 h. In comparison, Db molecules are processed more slowly (2 h) (Fig. 4). These results are in agreement with the relative transport rate of Kb and Db in RMA-S cells. IFN-γ treatment augments the synthesis of heavy chains causing more Kb and Db molecules to be transported to the cell surface of RMA and RMA-S cells. The rate of transport of Kb and Db molecules is not affected by IFN-γ treatment in RMA and RMA-S cells. In contrast, no Kb molecules are detected in CMT.64 cells using the 142.23.3 mAb. This mAb, as indicated earlier, may not recognize the unassembled and peptide-free heavy chains of Kb molecules (Fig. 4). To address this issue, we have used a rabbit anti-exon 8 serum directed against a conformation-independent epitope recognizing a peptide in the cytoplasmic tail of H-2Kb.

Figure 3. Effect of β2m on the CTL response against CMT.64 cells. Infected CMT.64 cells were superinfected with vaccinia and vaccinia-β2m recombinant (Vac/Vb2), vaccinia and VSV (Vac/VSV), or vaccinia-β2m VSV (Vb2-VSV) in FBS-free media (MOI 3) for up to an additional 12 h. (Inset) The level of β2m synthesized is shown after immunoprecipitation with the anti-hBzm rabbit serum. CMT.64 cells treated with peptide VSV-N52-59 at 500 pM for 2 h (CMT+p) was used as the positive control, whereas mock-treated CMT.64 cells (CMT+−−−) were used as the negative control. Radioactivity released is the average of quadruplicate wells. Spontaneous release did not exceed 16%.
molecules (40) to detect and follow the processing of K\textsuperscript{b} molecules in uninduced or IFN-\(\gamma\)-induced CMT.64 cells. In uninduced CMT.64 cells, K\textsuperscript{b} molecules are detectable early after synthesis (Fig. 5, 0-, 0.5-, and 1-h chase time), but are unstable and mostly degraded after 8-h chase with very few molecules processed to a higher molecular weight (Fig. 5, 8-h chase time). In induced cells, K\textsuperscript{b} molecules are synthesized in higher amounts and a greater proportion of the molecules are processed to a higher molecular weight (Fig. 5). We cannot explain the decrease in the amount of material immunoprecipitated by this antiserum during the chase. A loss or degradation of the epitope recognized by the antiserum during transport is possible. Furthermore, D\textsuperscript{b} molecules are also synthesized and are then degraded or denatured (Fig. 4). In uninduced CMT.64 cells, no processed D\textsuperscript{b} molecules can be detected even after 4-h chase. Only treatment with IFN-\(\gamma\) results in higher expression, increased transport, and increased transport rate of K\textsuperscript{b} and D\textsuperscript{b} molecules in CMT.64 cells. Thus, components necessary for the assembly and transport of K\textsuperscript{b} heavy chains and \(\beta\textsubscript{2}m\) are induced by IFN-\(\gamma\) in CMT.64 cells, whereas similar induction does not significantly alter the transport of D\textsuperscript{b} and K\textsuperscript{b} in RMA or RMA-S cells. This indicates that CMT.64 cells are likely deficient in components necessary for MHC class I assembly which differ from the TAP-2 defect in RMA-S cells.

Next, in order to assay the function of the MHC class I molecules, the CTL recognition of CMT.64 cells IFN-\(\gamma\)-induced or uninduced and RMA-S cells treated with exogenous peptides was examined (Fig. 6). In a dose-dependent manner, RMA-S and CMT.64 IFN-\(\gamma\)-treated cells were 10,000 times more sensitive than CMT.64 cells to killing by specific CTL after 2-h treatment with exogenous peptides. These results provide evidence for the low expression of peptide-receptive MHC class I molecules on the surface of uninduced CMT.64 cells. In the dose-response on RMA-S cells, a maximum of 15,000 peptide molecules per cell were needed to achieve 50% killing by specific CTL, whereas a lower threshold of 150 molecules per cell resulted in the release of 5-10% of \(^{51}Cr\). These data may be explained by a high amount of receptive molecules or high affinity of the MHC class I molecules for the peptide on the surface of RMA-S and IFN-\(\gamma\)-induced CMT.64 cells. Under the conditions of our assay, where there is no exogenous \(\beta\textsubscript{2}m\), the exogenously added peptides likely stabilize the empty K\textsuperscript{b} molecules which arrive at the cell surface of RMA-S before they dissociate from \(\beta\textsubscript{2}m\) (37, 45). The low amount of empty K\textsuperscript{b} transported in uninduced CMT.64 cells would explain the difference in sensitization to exogenous peptides.
The results shown in Figs. 2–6 indicate that despite its lower expression, β2m alone is not responsible for the lack of antigen presentation in CMT.64 cells. In addition, K\(^b\) and D\(^\alpha\) molecules are synthesized in these cells but very few are transported to the cell surface where they bind exogenously added peptides. Besides heavy and light chains, peptides are necessary for the efficient assembly of MHC class I molecules in the ER (10–12, 27). We have looked at the possibility that the absence of components responsible for the generation and transport of these peptides within the ER may be responsible for the CMT.64 phenotype.

A putative peptide transporter, presumed to be composed of a heterodimer of two half-ABC type transporters called TAP-1 and -2, has been implicated in translocating peptides into the ER for MHC class I assembly (15, 16). To characterize the difference of phenotypes between RMA-S and CMT.64 cells, the expression of TAP-1 and -2 genes in these cell lines was examined. In Fig. 7, Northern blot analysis shows that uninduced CMT.64 cells do not express a detectable amount of TAP-1 and -2 mRNA and that the amount of these mRNAs is highly increased after IFN-γ treatment of these cells. In addition, we show that no major difference exists between TAP-1 and -2 gene expression in RMA-S and RMA cells (Fig. 7) and that IFN-γ treatment only marginally affects TAP-1 and -2 expression in these cells. The amount of actin mRNA gives an indication of the near equal amount of mRNA loaded on the gel for Northern blotting. The IFN-γ inducibility of TAP-1 and -2 has been previously demonstrated in mouse tissues (46), however, this has not been examined in RMA, RMA-S, or CMT.64 cells before this present study. The results reported here show that the TAP-1 and -2 genes are IFN-γ inducible in CMT.64 cells and to a lesser degree in RMA and RMA-S cells. The absence of TAP-1 and -2 mRNA expression in CMT.64 cells likely causes a lack of antigenic peptides in the ER for binding to and assembly of MHC class I molecules. This results in the nonrecognition of VSV-infected CMT.64 cells. In contrast, RMA-S cells express a functional TAP-1 molecule that may aid peptides to cross the ER membrane. This would explain the assembly and transport of MHC class I in RMA-S cells and their CTL recognition after VSV infection. The lack of TAP-1 and -2 in uninduced CMT.64 cells may be one of the factors responsible for the phenotype of CMT.64 cells characterized by the
formation of unstable and inefficiently transported MHC class I complexes.

Before concluding that TAP deficiencies are the likely or only defects in CMT.64 cells, we decided to examine the presence of proteasome components in these cells. Viral peptides are thought to be generated in the cytoplasm by the proteasome (19–25) before crossing the ER membrane. The proteasome components are likely key players in antigen processing which could be absent in these cells. A rabbit anti-rat proteasome serum was used which recognizes the mouse proteasome. After immunoprecipitation of the proteasomes, the different component LMP produced in these mouse cells (Fig. 8) can be analyzed by two-dimensional gel electrophoresis. Two-dimensional gel analysis of immunoprecipitations reveals that the major components of the proteasome are not affected by IFN-γ treatment of CMT.64 cells but that seven components, including LMP-2 and -7, are missing in uninduced CMT.64 cells. According to the results of others (47), the proteins numbered 1 and 7 in Fig. 8 correspond to LMP-7 and -2, respectively. LMP-2, -7, and five other components of the proteasome are upregulated slightly by IFN-γ in RMA and RMA-S cells and are induced from a state of an undetectable expression to a higher detectable level of expression in IFN-γ–treated CMT.64 cells (Fig. 8). LMP-7 (Fig. 8) is particularly highly induced in CMT.64 cells treated with IFN-γ. These components are not recognized in any of the cell lines used in this study by nonimmune rabbit serum (data not shown). These results contrast the results of others which suggested that CMT.64 express a low level of all proteasome components (18) and these new results indicate that these induced proteasome components may affect the activity of the proteasome and allow the generation of the VSV-N peptides in induced CMT.64 cells. Recent data (48, 49) suggest that LMP-2 and -7 may not be necessary for influenza virus antigen presentation in mutant cells transfected with the TAP-1 and -2 genes.

Our results show that IFN-γ treatment in addition to inducing transcription of TAP-1 and -2 gene also upregulates the synthesis of seven components of the proteasomes, including LMP-2 and -7. Others describe that components in addition to LMP-2 and -7 are upregulated in Hela cell proteasomes by IFN-γ treatment (24, 47). However, as these cells are functionally wild type, the functional ramification of this regulation has not been addressed. Furthermore, as LMP-2 and -7 are first synthesized as precursor proteins which are cleaved into smaller products (47), it is possible that some of the five additional proteins missing from uninduced CMT.64 cells are precursor proteins of LMP-2 and -7.

Consideration of the accumulated data regarding antigen processing in RMA-S and CMT.64 cells leads to the contention that a functional TAP-1 protein homodimer alone may facilitate the transport of the VSV-N 52-59 peptide from the cytosol to the ER lumen where binding to the heavy chains takes place. An alternative explanation is that this peptide does not require a transporter for translocation across the ER membrane but is not generated in the CMT.64 cells. To more clearly define the defect affecting the recognition of VSV-infected CMT.64 cells by specific CTL, we have introduced the rat TAP-1 gene in CMT.64 cells. In Fig. 9, we show that VSV-infected TAP-1 positive CMT.64 cells are recognized by specific CTL. On the contrary, VSV-infected CMT.64 cells transfected with the vector DNA only are not recognized by specific CTL. This result indicates that TAP-1 alone is sufficient for restoration of VSV recognition in CMT.64 cells and explains the RMA-S phenotype and its apparent "leakiness" regarding VSV presentation (31, 32).
be sufficient for VSV presentation in RMA-S cells and in transfected CMT.64 cells and may form a homodimer capable of translocation of specific peptides into the lumen of the ER. Further studies are underway to define the role of transporter homodimers and heterodimers in peptide translocation during virus infection (Reid, G., R. Gabathuler, G. Kolaitis, and W. A. Jefferies, manuscript in preparation). In addition to transporters, the difference in the RMA-S and CMT.64 phenotype may be explained at one level by the higher amount of viral peptides generated in RMA-S cells. As reported recently, a more efficient protein degradation machinery is present in cells with proteasome containing all the LMP components (including LMP-2 and -7) (50, 51). This would cause a higher quantity of peptides to be expressed in cells expressing all the components of the proteasome. It is interesting to note that one may assume that a total repression of the expression of both LMPs and TAPs localized in the same region of class II may be sufficient for avoiding any expression of class I on the cell surface. This may be very important for some cancer cells (38, 52, 53) by providing a method by which tumor cells avoid immunosurveillance.

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