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, β_2-microglobulin (β_2m) 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 β_2m, the recognition by VSV-specific CTL is not restored by increasing the amount of β_2m 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 β_2m or K^b production. We find also 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 β_2-microglobulin (β_2m)[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

R. Gabathuler and G. Reid contributed equally to this work.

[1] *Abbreviations used in this paper:* β_2m, β_2-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 D^b 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 K^b 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 K^b 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 D^b molecules on CMT.64 cells and complement recognition by influenza-specific CTL (36). In addition, we have found that β_2m and the VSV-N 52-59 peptides added exogenously to these cells complement recognition by VSV-specific CTL restricted to K^b (37). The amount of β_2m 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 D^b and K^b 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 K^b 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 K^b 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 β_2m (hβ_2m) vaccinia recombinant were generous gifts from Dr. J. Yewdell (National Institutes of Health, Bethesda, MD).

Cell Lines and Antibodies. CMT.64 cells (H-2^b), graciously provided by Dr. L. M. Franks (Imperial Cancer Research Fund, London, UK), RMA, and RMA-S cells were maintained in DMEM supplemented with 10% heat inactivated FCS, 20 mM Hepes, 2 mM glutamine, and antibiotics. The mAbs used were as follows: 142-23.3 anti-H-2 K^b, 28-11-5s anti-H-2 D^b (α1 + α2), 28-14-8s, anti-H-2 D^b (α3), and BBM.1 against human β_2m (38). A rabbit antiserum against hβ_2m (39), against exon-8 of H-2K^b (40), and against rat proteasome (20) were also used.

Transfection. Transfection of CMT.64 cells with rat cDNA TAP-1 in the pHb APr-1 neo expression vector (kindly provided by Dr. G. Butcher, AFRC, Institute of Animal Physiology, Cambridge, UK) was achieved by lipofection (Lipofectin; GIBCO BRL, Gaithersburg, MD) using 10 μg of DNA. Selection was in 1 mg/ml G418 (GIBCO BRL). Positive clones were selected and screened by Northern blotting for expression of the rat TAP-1 gene. 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 hβ_2m (2.5 μg). Peptides were purchased from the University of Victoria, Peptide Synthesis Facility (Victoria, BC, Canada). The cells were subsequently removed from culture, washed, and incubated with 1:50 dilution of 142-23.3 ascites, or 200 μl of cell culture supernatant from 28-11-5s and 28-14-8s cells for 45 min on ice. After two washes, the cells were incubated with 100 μl of 1:20 dilution of goat anti–rabbit, or goat anti–mouse FITC–conjugated secondary antibody for another 45 min on ice. The samples were then fixed in paraformaldehyde (1.5% in PBS) and analyzed on a FACScan® cell sorter using the FACScan® 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 [^{35}S]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 $MgCl_2$, 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 ^{51}Cr per 10^6 cells for 2 h in RPMI 1640 supplemented with L-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-hβ_2m (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× and subsequently incubated at 10^4 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 5×10^6–1×10^7 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 4×10^6 cells/ml in a total volume of 5 ml in 6-well plates. The culture medium consisted of RPMI-1640 supplemented with 5×10^{-5} M 2-ME, 10% heat-inactivated FBS, sodium pyruvate, penicillin, streptomycin, L-glutamine, Hepes, sodium bicarbonate, and 50% NCTC-109. Cultures were incubated for 3 d at 37°C and 5% CO_2 in the absence of exogenous stimulation. The ^{51}Cr-release was measured by a compugamma counter (model 1282 CS; LKB Instruments, Gaithersburg, MD) and the specific ^{51}Cr-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 H_2O. 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 (Amersham Corp., Arlington Heights, IL) and U/V fixed before hybridization. The ^{32}P-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 transferase. Hybridization was carried out at 42°C in buffer containing 0.4 M Na_2HPO_4, 50% formamide, 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

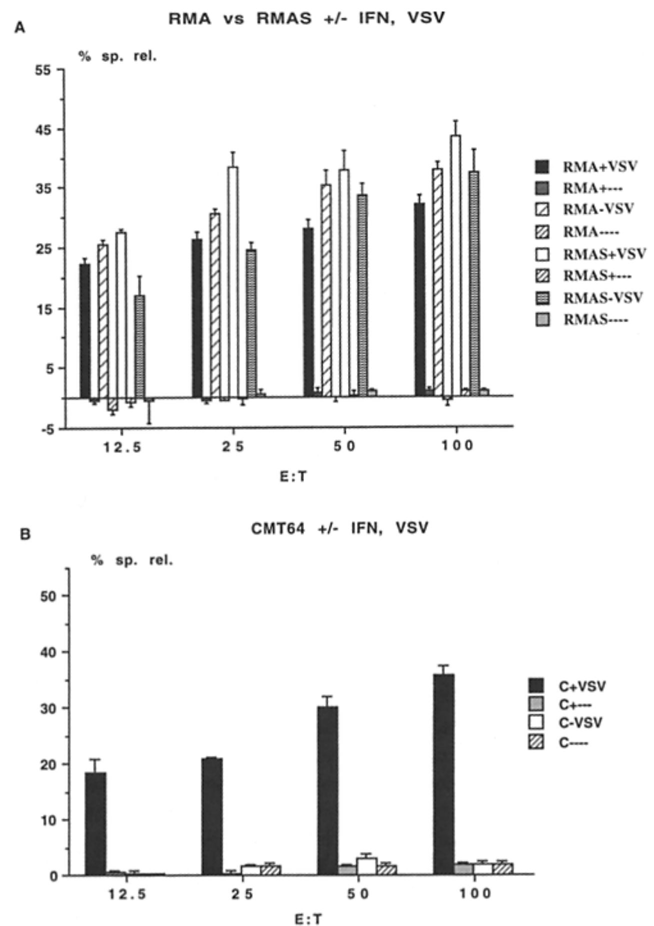

Figure 1. CTL recognition of VSV-infected RMA, RMA-S, CMT.64 IFN-γ induced or uninduced cells. Targets were treated with or without IFN-γ for 48 h before infection with VSV. (*A*) Representative experiment using RMA and RMA-S cells as targets. (*B*) Equivalent experiment with CMT.64 cells (*C*). All cells were infected with VSV at a MOI of 10 for 4 h. IFN-γ treatment is denoted with a + sign after the cell line designation. Spontaneous release did not exceed 15%.

Table 1. *Peptides (p), hβ_2m (β_2m), and IFN-γ (IFN) Treatment Modifies the Conformation of K^b and D^b Expressed on the Cell Surface of RMA, RMA-S, and CMT.64 Cells*

					Antibodies		
		Treatment			142-23.3	28-11-5s	BBM.1
	Cell lines	IFN	*p*	β_2m	K^b spec.	D^b spec.	hβ_2m spec.
Experiment 1							
	CMT 64	−	−	−	5*	3	ND
		−	+	+	8	5	ND
		+	−	−	26	58	ND
		+	+	+	35	53	ND
Experiment 2							
	RMA	+	−	−	346	430	6
		+	+	−	606	449	4
		+	−	+	438	549	406
		+	+	+	780	515	606
	RMA-S	+	−	−	12	4	14
		+	+	−	10	1	14
		+	−	+	10	2	76
		+	+	+	41	2	262
Experiment 3							
	RMA	−	−	−	164	173	1
		−	+	+	242	182	274
	RMA-S	−	−	−	12	2	1
		−	+	+	70	2	86

* Arbitrary fluorescense units.

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-γ treatment (Fig. 1 *A*), in comparison to VSV-infected CMT.64 cells which are not recognized by specific CTL unless induced by IFN-γ (Fig. 1 *B*). 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.

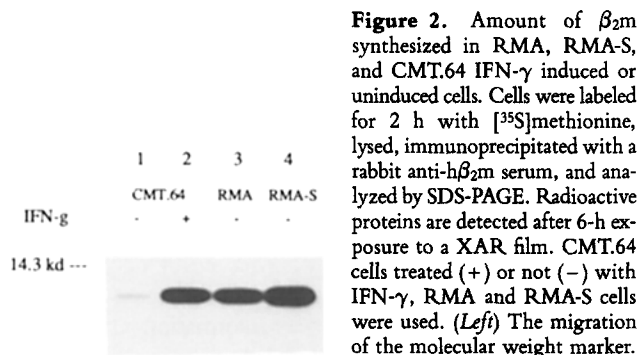

Figure 2. Amount of β_2m synthesized in RMA, RMA-S, and CMT.64 IFN-γ induced or uninduced cells. Cells were labeled for 2 h with [^{35}S]methionine, lysed, immunoprecipitated with a rabbit anti-hβ_2m serum, and analyzed by SDS-PAGE. Radioactive proteins are detected after 6-h exposure to a XAR film. CMT.64 cells treated (+) or not (−) with IFN-γ, RMA and RMA-S cells were used. (*Left*) The migration of the molecular weight marker.

Previous experiments have demonstrated that treatment of mutant cells with exogenous peptides and/or human β_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-γ were treated overnight with exogenous peptides VSV-N 52-59 at 50 μM in the presence or absence of human β_2m. VSV-N 52-59 peptides and β_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 β_2m binds to K^b and D^b molecules, which are detected by BBM.1 (anti–human β_2m mAb), and appears to stabilize heavy chains before they

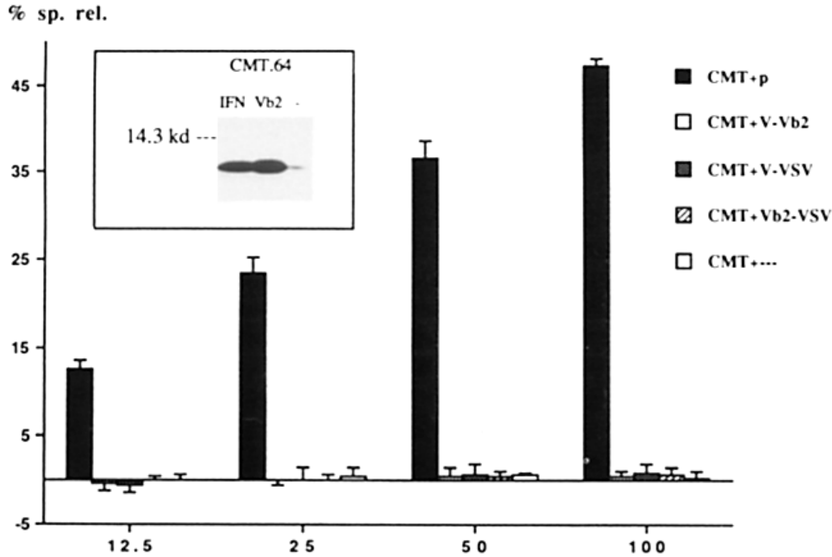

Figure 3. Effect of β_2m on the CTL response against CMT.64 cells. Infected CMT.64 cells were superinfected with vaccinia and vaccinia-β_2m recombinant (*V-Vb2*), vaccinia and VSV (*V-VSV*), or vaccinia-β_2m VSV (*Vb2-VSV*) in FBS-free media (MOI 3) for up to an additional 12 h. (*Inset*) The level of β_2m synthesized is shown after immunoprecipitation with the anti-hβ_2m 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%.

can disassemble at the cell surface. A stabilizing effect is not seen after CMT.64 treatment with peptides or β_2m alone. Additional treatment of CMT.64 cells with IFN-γ is required for high expression of K^b and D^b 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 β_2m 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 β_2m (Fig. 2, lane *1*). IFN-γ–induced CMT.64 cells express a much higher amount of β_2m, which is comparable to the level expressed in RMA and RMA-S cells (Fig. 2, lanes *2–4*). To investigate the effect of β_2m in CMT.64 cells, we have used a recombinant vaccinia virus to increase the amount of endogenous β_2m (Fig. 3, *inset*). Elevating the amount of β_2m 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 β_2m does not induce presentation of VSV-N peptides in the context of K^b molecules. The CMT.64 antigen-processing phenotype is not caused by the low amount of endogenous β_2m.

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 K^b and D^b 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 K^b, 142.23.3 mAb was used and for D^b, 28-14-8s, an α3-specific mAb was used) (Fig. 4). Despite a similar amount of K^b molecules synthesized in RMA and RMA-S cells (Fig. 4, 0-h chase time), only low amounts of K^b are processed to a higher molecular weight form, indicative of the level of transport which accounts for the surface expression of K^b 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 β_2m is immunoprecipitated with K^b molecules than with D^b 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 K^b molecules, whereas the mAb 28-14-8s recognizes the α3 region of D^b 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 K^b molecules allowing them to go to the cell surface. Also, peptides with lower affinity for K^b molecules may bind and aid the molecules to assemble and go to the cell surface where they dissociate. Much fewer mature processed D^b molecules are detected in RMA-S cells after 4-h chase (Fig. 4). This may indicate a lower affinity of D^b for β_2m and/or fewer peptides available for D^b binding. In RMA cells, K^b molecules are processed within 1 h. In comparison, D^b molecules are processed more slowly (2 h) (Fig. 4). These results are in agreement with the relative transport rate of K^b and D^b in RMA-S cells. IFN-γ treatment augments the synthesis of heavy chains causing more K^b and D^b molecules to be transported to the cell surface of RMA and RMA-S cells. The rate of transport of K^b and D^b molecules is not affected by IFN-γ treatment in RMA and RMA-S cells. In contrast, no K^b 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 K^b 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-$2K^b$

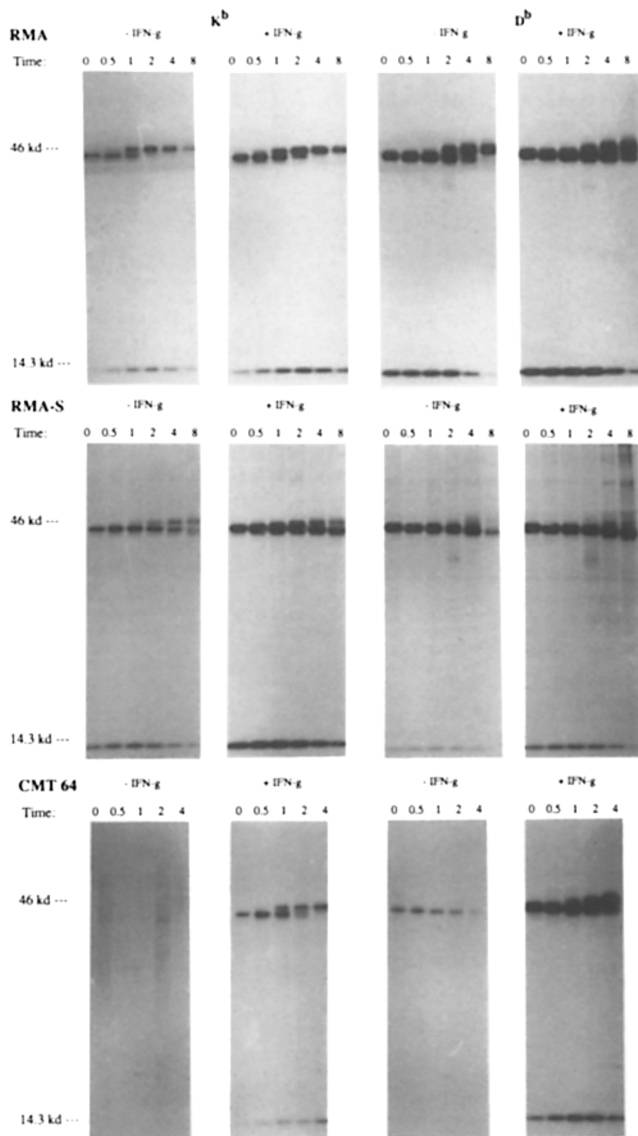

Figure 4. Intracellular transport of MHC class I molecules, D^b and K^b. Cells were labeled with [^{35}S]methionine and chased in an excess of cold methionine for the times indicated in hours. Solubilized antigens were immunoprecipitated with 142.23.3 mAbs for K^b or 28-14-8s mAbs for D^b. (*Top*) Treatments; (*left*) migration of the molecular markers. Coimmunoprecipitation of the heavy chains (*46 kd*) and β_2m (12 kD) can be seen for all cells except for CMT.64 uninduced cells. Radioactive proteins are detected after 8-d exposure for RMA-S cells and 4 d for RMA and CMT.64 cells to a XAR film.

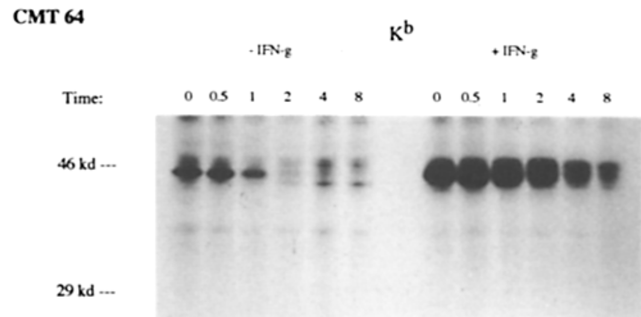

Figure 5. Intracellular transport of free and assembled forms of K^b molecules in uninduced and IFN-γ-induced CMT.64 cells. Cells were labeled with [^{35}S]methionine and chased in an excess of cold methionine for the times indicated in hours. Solubilized antigens were immunoprecipitated with a rabbit anti-exon 8 of H-2K^d serum recognizing the cytoplasmic tail of free and assembled K^b heavy chains. (*Top*) Treatments; (*left*) migration of the molecular weight markers. Radioactive proteins are detected after 4-d exposure to a RPN-30 film (Amersham Corp.).

molecules (40) to detect and follow the processing of K^b molecules in uninduced or IFN-γ-induced CMT.64 cells. In uninduced CMT.64 cells, K^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^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^b molecules are also synthesized and are then degraded or denatured (Fig. 4). In uninduced CMT.64 cells, no processed D^b molecules can be detected even after 4-h chase. Only treatment with IFN-γ results in higher expression, increased transport, and increased transport rate of K^b and D^b molecules in CMT.64 cells. Thus, components necessary for the assembly and transport of K^b heavy chains and β_2m are induced by IFN-γ in CMT.64 cells, whereas similar induction does not significantly alter the transport of D^b and K^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-γ 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-γ-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-γ-induced CMT-64 cells. Under the conditions of our assay, where there is no exogenous β_2m, the exogenously added peptides likely stabilize the empty K^b molecules which arrive at the cell surface of RMA-S before they dissociate from β_2m (37, 45). The low amount of empty K^b transported in uninduced CMT.64 cells would explain the difference in sensitization to exogenous peptides.

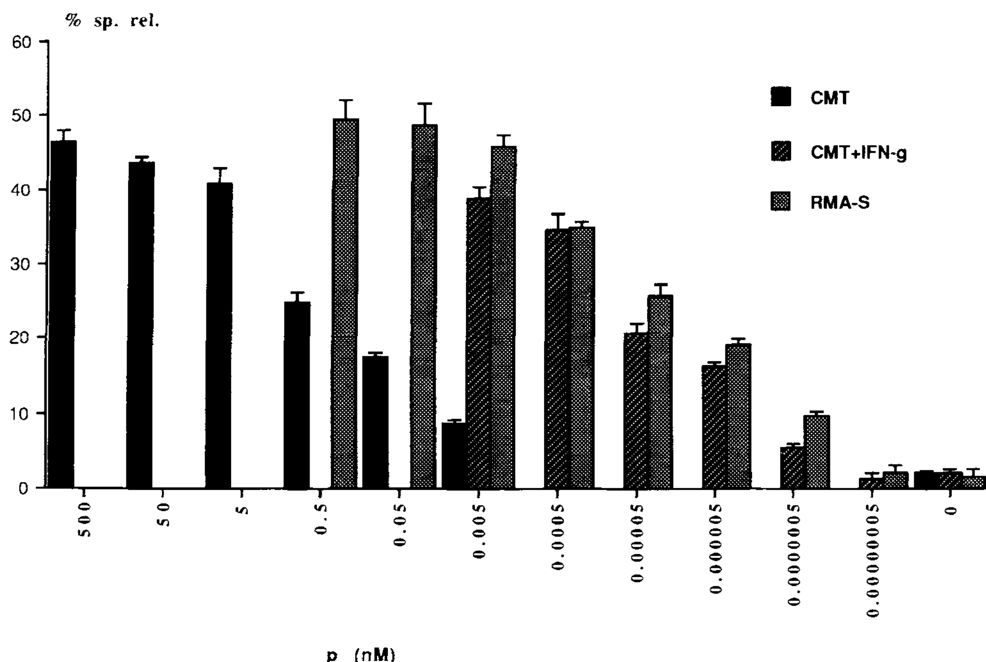

Figure 6. VSV-N 52-59 peptide dose–response in CTL recognition. CMT.64 cells (*CMT*), CMT.64 + IFN-γ (*CMT + IFN-γ*), and RMA-S cells (*RMA-S*) were treated with peptide N52-59 at the concentrations indicated. The radioactivity released by specific CTL recognition and lysis was measured and represented as indicated in Materials and Methods. Radioactivity released is the average of quadruplicate wells. Spontaneous release did not exceed 13%.

The results shown in Figs. 2–6 indicate that despite its lower expression, β_2m alone is not responsible for the lack of antigen presentation in CMT.64 cells. In addition, K^b and D^b 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

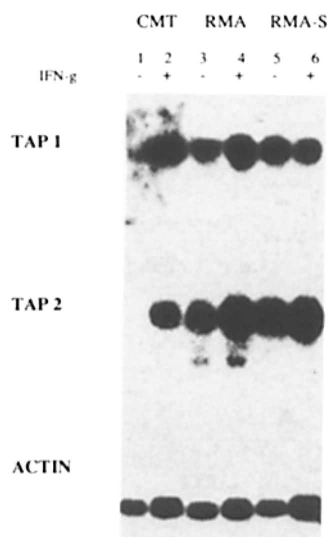

Figure 7. Northern blot analysis of total cellular RNA from RMA, RMA-S, and CMT.64 IFN-γ induced and uninduced cells. 10 μg of cytoplasmic RNA from CMT.64 (lanes *1* and *2*), RMA (lanes *3* and *4*), and RMA-S (*lanes 5* and *6*) cells IFN-γ-induced (+) or uninduced (−) were analyzed. TAP-1, TAP-2, and β-actin probes were hybridized with the membrane. The radioactivity bound to specific RNA sequences is detected after overnight exposure of the membrane to a XAR film.

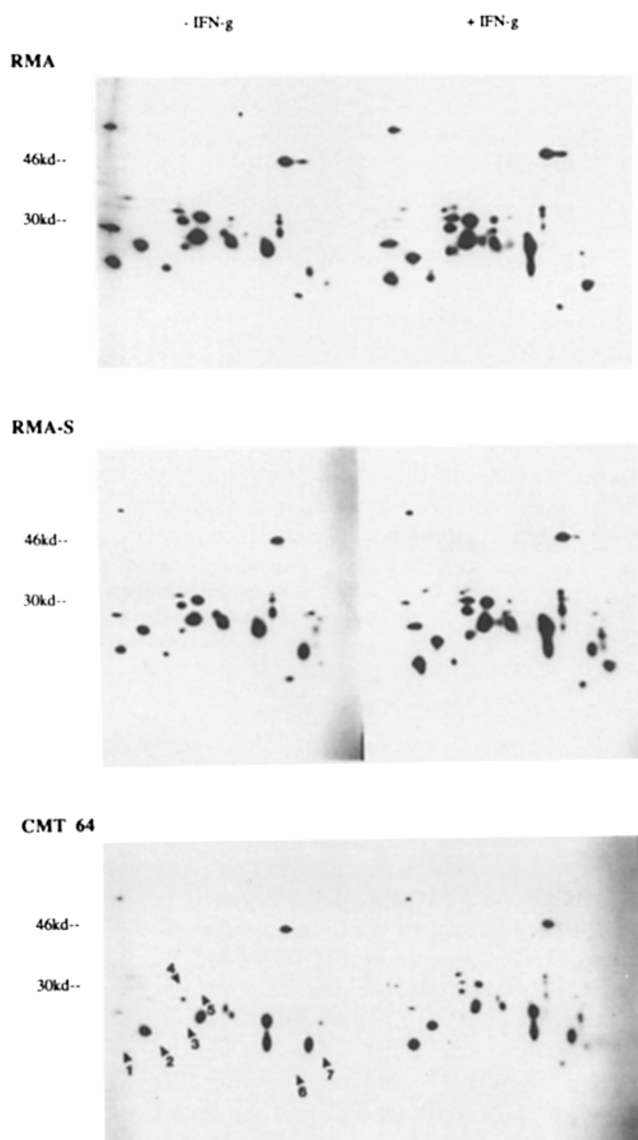

Figure 8. Two-dimensional gel analysis of proteasome components from RMA, RMA-S, and CMT.64 IFN-γ–induced or uninduced cells. Cells were labeled for 2 h with [^{35}S]methionine. Solubilized antigens were immunoprecipitated with a rabbit anti-rat proteasome serum and analyzed after isoelectric focusing in a first dimension and 10–15% SDS-PAGE in a second dimension. The radioactive proteins are detected after 10-d exposure to a XAR film. (*Top*) Treatment of the cells; (*right*) acidic side of the gel; and (*left*) basic side of the gel. The migration of the molecular weight markers is indicated on the left. The missing proteins are indicated by an arrow and are numbered. Proteins numbered 1 and 7 correspond to LMP-7 and LMP-2, respectively.

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). TAP-1 alone may

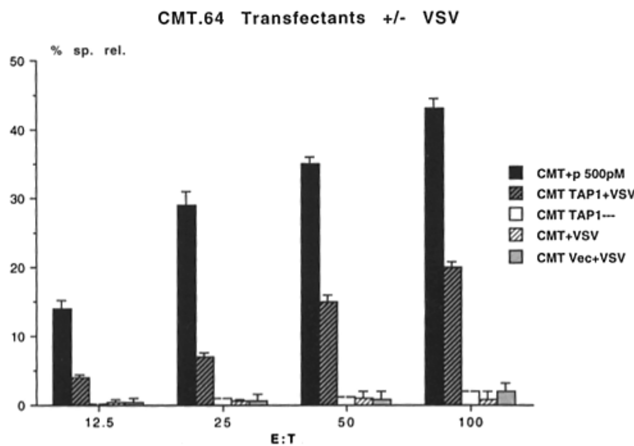

Figure 9. CMT 64 (*CMT*), CMT 64 transfected with TAP-1 (*CMT TAP1*), and CMT.64 transfected with the vector only (*CMT Vec*) were infected with or without VSV for 8 h at a MOI of 5, or treated with N52-59 peptide for 2 h at 500 pM (50% dose-response). Spontaneous release did not exceed 12%.

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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