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P-Glycoprotein Plays an Insignificant Role in the Presentation of Antigenic Peptides to CD8$^+$ T Cells

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ABSTRACT

Most antigenic peptides presented to CD8$^+$ T cells are generated from cytosolic precursors and are translocated by TAP into the endoplasmic reticulum, where they associate with MHC class I molecules. TAP-deficient cells exhibit a limited capacity to deliver peptides from cytosolic proteins to class I molecules. One candidate for an alternative peptide transporter is P-glycoprotein, which transports numerous substances, including peptides, across membranes. Elevation of P-glycoprotein expression is partially responsible for the resistance developed by neoplasias to chemotherapeutic drugs. Overexpression of P-glycoprotein has been reported to enhance the expression of class I molecules. Here, we investigated the role of P-glycoprotein in the generation of peptide-MHC complexes. We were unable to detect P-glycoprotein-mediated transport of synthetic peptides into the endoplasmic reticulum of either T2 cells (TAP-deficient) infected with a recombinant vaccinia virus (rVV) expressing P-glycoprotein or drug-resistant cells in which TAP is inactivated by a peptide from the herpes simplex virus ICP47 protein. Expression of rVV-encoded P-glycoprotein in T2 cells was unable to enhance cell surface expression of any of three MHC class I allomorphs tested. rVV-mediated expression of P-glycoprotein enabled T2 cells to produce limited amounts of class I-peptide complexes from cytosolic antigens, but this was not blocked by a drug that inhibits its transporter function, and a similar degree of presentation was mediated by functionally inactive mutated forms of P-glycoprotein. Thus, this was a nonspecific effect that we attributed to diminished membrane integrity resulting from P-glycoprotein overexpression. Taken together, our findings cast serious doubts that P-glycoprotein is a biologically significant transporter of cytosolic peptides.

INTRODUCTION

T_{CD8+}[3] recognize peptides, usually 8–10 residues in length, bound to MHC class I molecules. The majority of peptides are generated from cytosolic precursors and are transported into the ER, where they associate with nascent class I molecules. TAP-deficient cells exhibit an ~10-fold decrease in the expression of conformed class I molecules on the cell surface. The majority of peptides presented by TAP-deficient cells are derived from proteins delivered to the ER by signal sequences, but the cells also maintain a limited capacity to present peptides from cytosolic proteins (1).

One candidate peptide transporter is P-glycoprotein. Like TAP, P-glycoprotein is a member of a large family of integral membrane transporters referred to as ATP-binding cassette proteins. Each member of the family has at least one homologous domain that binds and hydrolyzes ATP. ATP hydrolysis drives transport of the wide variety of substrates handled by the various family members (for review see Ref. 2). P-glycoprotein is the closest known relative of TAP in mammalian cells. It is able to transport numerous cytosolic substrates across the plasma membrane, including peptides (3–7). The normal function of P-glycoprotein in cells is not well established, but its expression is often associated with the resistance developed by tumor cells to cytotoxic drugs during the course of chemotherapy (reviewed in Ref. 8; Ref. 9). Such resistance extends well beyond the selecting agent to include numerous drugs, and so it is termed MDR.

The potential involvement of P-glycoprotein in antigen processing was suggested by Masci *et al.* (10), who reported that: (*a*) human cells selected for MDR *in vitro* or expressing P-glycoprotein from transfected cDNA bound higher levels of some mAbs specific for HLA class I-molecules than did nonselected or control transfected cells; and (*b*) it was possible to reduce HLA-specific mAb binding to cells expressing elevated levels of P-glycoprotein (but not normal cells) or even a subset of PBLs by incubating cells for 12 h with verapamil, a commonly used inhibitor of P-glycoprotein.

On the basis of these findings, it was proposed that P-glycoprotein may play an important role in providing peptides to class I molecules, particularly in multidrug-resistant tumor cells or normal cell types with high levels of endogenous P-glycoprotein expression (10).

Here, we have investigated the ability of P-glycoprotein to contribute to antigen processing. We find no evidence that P-glycoprotein is able to either transport peptides into the ER, facilitate the cell surface expression of newly synthesized class I molecules, or specifically enable the presentation of viral antigens to specific T_{CD8+}.

MATERIALS AND METHODS

Cells and Viruses. The antigen processing-deficient human cell line T2 (11) and human epidermoid carcinoma KB-3-1 and KB-V1 cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 7.5% (v/v) fetal bovine serum. The multidrug resistant line KB-V1 (expressing P-glycoprotein) was maintained in 1 μg/ml vinblastine, which was removed from the medium at least 4 days before an experiment. High levels of P-glycoprotein are maintained for at least a month following the removal of vinblastine. rVVs were propagated in thymidine kinase-deficient human 143B osteosarcoma cells. rVVs expressing K^d, TAP1, TAP2, TAP[1 + 2], P-glycoprotein (MDR1), NP, $NP_{M147-155}$, ICP47, and T7 under the control of the early/late VV p7.5 promoter have been described (12, 13). Further rVVs were used to express P-glycoprotein [MDR1(T7)] and P-glycoprotein mutants (NM-D555N, CM-D1200N, and DM-D555N + D1200N), under the control of the T7 promoter (14). The function of rVV-encoded wild-type P-glycoprotein has been demonstrated (14).

Mice. Six- to 8-week-old BALB/c (H-2^d) mice were obtained from TACONIC (Germantown, NY). Mice were immunized with 10^6 plaque forming units of rVV by i.v. injection.

Cytotoxicity Assay. Target cells were infected with rVVs for 4 h at 37°C and labeled for 1 h at 37°C with $Na^{51}CrO_4$. T_{CD8+} were generated from splenocytes derived from animals immunized with rVV 2–6 weeks previously by 7-day *in vitro* stimulation with influenza virus or VV-infected autologous splenocytes. Target cells were incubated with effector cells for 6 h at 37°C and

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[3] The abbreviations used are: T_{CD8+}, CD8$^+$ cell(s); ER, endoplasmic reticulum; TAP, transporter associated with antigen processing; MDR, multidrug resistance; mAb, monoclonal antibody; HLA, human lymphocyte antigen; PBL, peripheral blood lymphocyte; rVV, recombinant vaccinia virus; VV, vaccinia virus; SLO, streptolysin O; m.o.i., multiplicity of infection.

the amount of ^{51}Cr released into the supernatant was determined by γ-counting. Data are expressed as percentage specific release, defined as:

$$[(\text{Experimental cpm} - \text{spontaneous cpm})/(\text{total cpm} - \text{spontaneous cpm})] \times 100$$

Cytofluorography. Cells were incubated for 30 min at 0°C with fluorescein-conjugated antibodies specific for MHC class I molecules [W6/32 (Biodesign International, Kennebunk, ME) or SF1.1.1 (Pharmingen, San Diego, CA)] or unconjugated mAbs specific for P-glycoprotein (MRK-16; Ref. 15), TAP (148.3; Ref. 16), or HLA-B5 (HB-178; ATCC, Manassas, VA); washed; and incubated for 30 min at 0°C with fluorescein-conjugated rabbit antimouse immunoglobulin (DAKO, Carpinteria, CA) to detect binding of the unconjugated mAb. Cells were washed and resuspended in PBS containing 10 μg/ml ethidium homodimer (Molecular Probes, Portland, OR) and analyzed using a FACScan (Becton Dickinson, San Jose, CA). Nonviable cells were excluded from analysis. To detect expression of TAP, cell were first fixed for 15 min at room temperature with 3% paraformaldehyde in PBS and then permeabilized by inclusion of 0.1% saponin in all subsequent staining and washing solutions.

Peptide Translocation. Modified antigenic peptides from influenza virus HA and NP (LYQNNGTYV and TYNRTARLV; synthesized by the Biological Resources Branch, National Institute of Allergy and Infectious Diseases, Rockville, MD) containing Tyr residue for labeling with ^{125}I and N-X-T glycosylation consensus sequence were used as reporter peptides. Peptides were iodinated by the chloramine T method and separated from unreacted ^{125}I by passage through a Dowex column. Cells were permeabilized with SLO (Murex Diagnostics, Norcross, GA), incubated with ^{125}I-labeled peptide for 20 min, and solubilized with buffer containing 1% Triton X-100. Samples were centrifuged, and soluble material was incubated with Sepharose-bound Con A to recover glycosylated material. After washing, the amount of bound peptide was determined by α-counting.

RESULTS

Expression Systems. We used two systems to analyze the role of P-glycoprotein in the generation of class I-peptide complexes. The majority of the experiments utilize recombinant vaccinia viruses to express transporter proteins in the T2 cell line. T2 cells express neither P-glycoprotein (17)[4] nor MDR-associated protein (17), a related transporter. T2 cells possess a single copy of chromosome 6 (the location of the HLA complex) derived from 721.174 cells, a γ-irradiated EBV-transformed human lymphoid cell line immunoselected for low cell surface expression of class I molecules (18). T2 cells express normal levels of class I mRNA, but assembly of class I molecules is compromised by the absence of *TAP1* and *TAP2* genes, which are located in the ~1-Mbp region deleted as a result of irradiation. Cells were infected with rVVs expressing P-glycoprotein or other proteins under the control of the p7.5 VV promoter. This is the promoter used in most antigen processing and presentation studies. To express ~10-fold higher levels of P-glycoprotein, we also infected cells with rVVs encoding P-glycoprotein under the control of the T7 promoter. In these experiments, cells were coinfected with a rVV expressing the T7 polymerase under control of the p7.5 promoter (19). To determine the requirement for a functional P-glycoprotein, we used rVVs expressing three mutated forms of the protein under the control of the T7 promoter, termed CM, NM, and DM. The introduced mutations alter one or both of highly conserved Asp residues located, respectively, within Walker B regions of either NH_2-terminal (D555N) or COOH-terminal (D1200N) ATP binding/utilization sites that are believed to be involved in binding to Mg^{2+}. These mutations, singly or in combination, eliminate ATP hydrolysis and drug pumping by P-glycoprotein.[5]

To examine the effect of MDR on expression of MHC class I antigen presentation, we used human KB cells grown in the cytotoxic drug vinblastine (KB-V1 cells). The complicating effects of vinblastine on antigen processing or presentation were minimized by removing cells from vinblastine at least 4 days prior to their use. MDR is maintained for at least 2 weeks following the removal of the selecting drug. Cytofluorographic analysis of fixed and permeabilized cells revealed that drug selection did not alter the amount of TAP1 detected by the 148.3 mAb (data not shown). To study the antigen processing activity of P-glycoprotein in the absence of TAP in these cells, we used the ICP47 protein of herpes simplex virus to inhibit TAP translocation of peptides. ICP47 was used either as a protein synthesized by a rVV or as a synthetic peptide corresponding to residues 1–35 of the protein that has been reported to function as efficiently as the full-length protein (20). The use of ICP47 for this purpose hinges on it not affecting any peptide translocating activity that P-glycoprotein may have. Because ICP47 is highly species specific, being unable to block mouse TAP (21), which is far more homologous to human TAP than is P-glycoprotein, this seemed a reasonable assumption. In support of this conclusion, we found that expression of ICP47 from rVV does not affect the function of P-glycoprotein, as measured by export of rhodamine-123 from cells, nor does the synthetic peptide, even when present at 100 μM, after the ATPase activity of P-glycoprotein in membranes (data not shown).

Reporter Antigenic Peptides Are Not Translocated across the ER Membrane by P-Glycoprotein in TAP-deficient Cells. P-glycoprotein is predominantly expressed in the plasma membrane. Like most membrane proteins, it is synthesized in the ER and transported to the plasma membrane through the Golgi complex (22–26). To study the ability of P-glycoprotein to translocate peptides into the ER, we measured the capacity of permeabilized cells to glycosylate two radioiodinated synthetic peptides (LYQNNGTYV and TYNRTARLV) known to be transported by TAP. The peptides contain a N-linked glycosylation site and are glycosylated upon delivery of the ER, the sole site of N-linked glycosylation in cells. Glycosylation is detected by the recovery of radioactivity on Con A-Sepharose beads incubated with detergent extracts of cells.

TAP or P-glycoprotein was expressed by infecting T2 cells with the appropriate rVV. As we demonstrated previously (13), expression of both TAP subunits results in easily detected glycosylation over background values obtained by expression of TAP1 alone (Fig. 1; data shown are for LYQNNGTYV, similar findings were obtained using TYNRTARLV). Expression of large amounts of P-glycoprotein by use of the T7 expression system resulted in a marginal increase in glycosylation over expression of TAP1 alone. This small signal is not, however, due to the transport properties of P-glycoprotein because a similar signal was obtained from cells expressing the nonfunctional forms of P-glycoprotein.

The ability of endogenously expressed P-glycoprotein to transport iodinated peptides into the ER was examined using the drug-sensitive human KB-3-1 cell line and its vinblastine-selected multidrug-resistant variant KB-V1, which expresses high levels of P-glycoprotein. To distinguish TAP-dependent from TAP-independent peptide transport into the ER, we added a synthetic peptide corresponding to residues 1–35 of ICP47. This peptide has been shown to be a potent inhibitor of TAP-mediated peptide transport. As seen in Fig. 1 *B*, ICP47 was able to reduce the peptide transport to background levels. Together with the results obtained with T2 cells, these findings indicate that P-glycoprotein is unable to transport the indicator peptides into the ER at detectable levels.

Effect of P-Glycoprotein on the Cell Surface Expression of MHC Class I Antigens. It is possible that P-glycoprotein is able to transport into the ER a subset of TAP-transported peptides excluding

[4] Unpublished observations.

[5] C. A. Hrycyna, M. Ramachandra, I. Pastan, and M. M. Gottesman. Both ATP binding sites of human P-glycoprotein are essential but non-identical, manuscript in preparation.

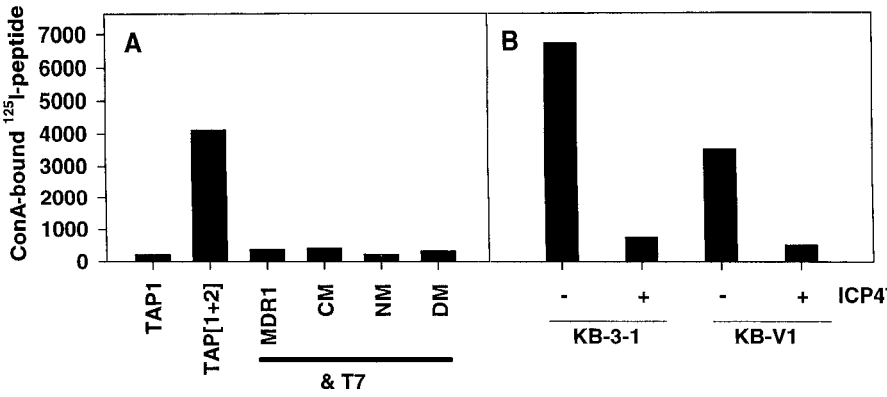

Fig. 1. Peptide translocation. *A*, ^{125}I-labeled HA peptide (LYQNNGTYV) was incubated with SLO-permeabilized T2 cells infected for 5 h with the rVVs indicated. *B*, ^{125}I-labeled HA peptide (LYQNNGTYV) was incubated with SLO-permeabilized KB-3-1 and KB-V1 cells in the absence or presence of synthetic ICP47 peptide (20 μM) as indicated. Peptides that were N-glycosylated following transport into the ER were isolated with Con A-Sepharose and quantitated by γ-counting. Similar results were obtained using TYNRTRALV.

the two indicator peptides used above. It is also plausible that any effects of P-glycoprotein on class I cell surface expression are unrelated to its ability to facilitate peptide transport into the ER. For example, the Golgi complex possesses peptide-receptive class I molecules (27), and P-glycoprotein may transport peptides into the Golgi complex but not the ER. Therefore, we examined the effect of P-glycoprotein expression on the expression of MHC class I molecules.

In contrast to the findings of Masci *et al.* (10), we did not detect a significant difference between the expression of mAb W6/32-reactive class I molecules on the surface of vinblastine selected-KB cells relative to nonselected cells (data not shown). We further explored the effect of P-glycoprotein on MHC class I expression using T2 cells. T2 cells naturally express HLA A2 and HLA B5 class I molecules. The cells used also express mouse H-2 K^d molecules from a transfected cDNA. Expression of B5 and K^d is low in these cells due to the absence of TAP (Fig. 2). In contrast, A2 expression is less severely compromised, as indicated by the staining with W6/32, a HLA A,B,C panreactive mAb. This is due to the preference of A2 for hydrophobic peptides, which can be obtained from ER insertion sequences in a TAP-independent manner. As expected, infection of T2 cells with VV-TAP [1 + 2] results in the enhanced cell surface expression of B5. The effect of TAP is not significantly affected by inclusion of verapamil during the infection period.

In contrast to TAP, expression of P-glycoprotein from the T7 promoter had no discernible effect on HLA B5 expression relative to cells infected with a control rVV or to the nonfunctional CM mutant. The background levels of B5 expression in MDR1-infected cells were not further decreased by verapamil. In this experiment, the cell surface expression of P-glycoprotein was demonstrated cytofluorographically using a P-glycoprotein-specific mAb. Similarly, TAP but not P-glycoprotein was able to enhance expression of either K^d or W6/32 reactive class I molecules, which include both B5 and A2. These findings demonstrate that VV-expressed P-glycoprotein is not able to provide a sufficient quantity of peptides to detectably enhance cell surface expression of three class I molecules that bind largely non-overlapping spectra of peptides.

Inefficient Rescue of Antigen Presentation in TAP-deficient T2 Cells by Overexpression of P-Glycoprotein. The most sensitive method of measuring of cell surface expression of class I-peptide complexes is to test cells for lysis by T_{CD8+}, which are capable of lysing cells expressing very few copies of a given complex. To determine whether P-glycoprotein is able to provide low levels of

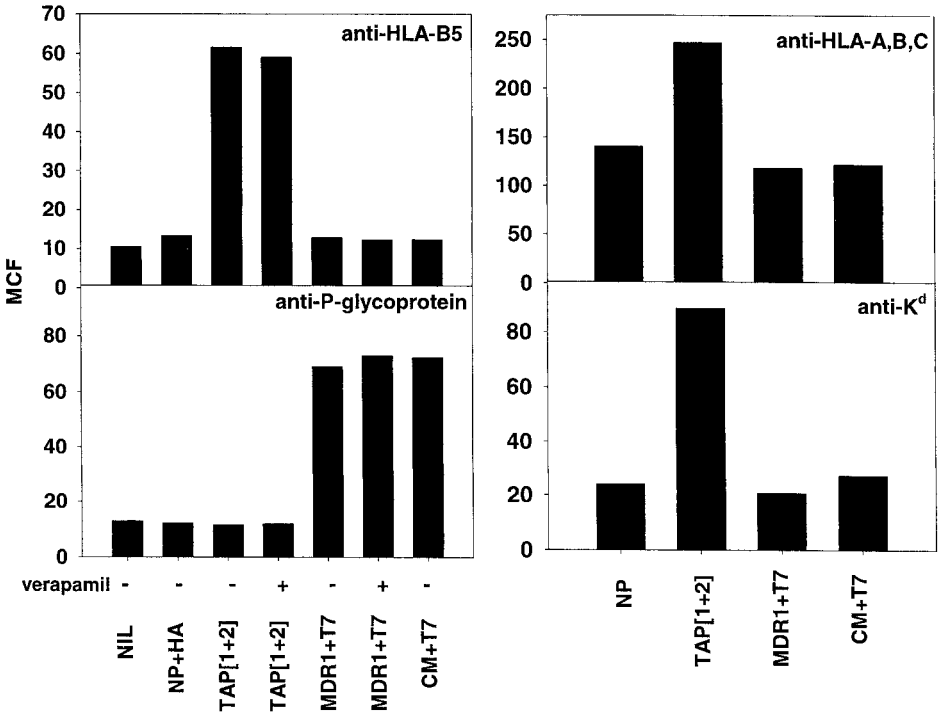

Fig. 2. Cell surface expression of class I molecules and P-glycoprotein in T2-K^d cells. T2-K^d cells infected overnight (13 h) with the rVVs expressing the indicated proteins were tested for binding to nonconjugated mAbs specific for HLA B5 or P-glycoprotein (*left*) or fluorescein-conjugated W6/32 (specific for HLA, A,B,C) molecules or SF1.1.1 (specific for K^d; *right*). Columns, the log mean channel fluorescence (*MCF*) of viable cells.

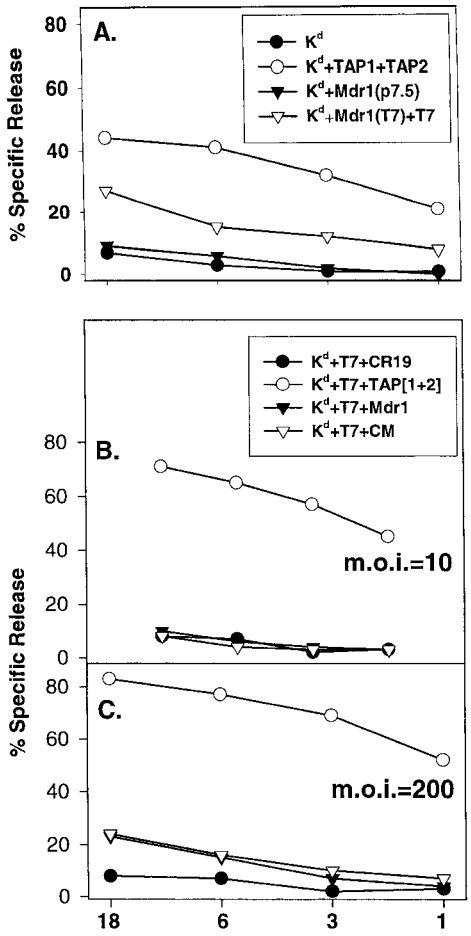

Fig. 3. Rescue of antigen presentation in TAP-deficient T2 cells. T2 cells infected with the indicated rVVs for 4 h before testing in a 6-h cytotoxicity assay using secondary *in vitro* restimulated VV-specific effector cells at the effector to target ratio indicated. *A*, rVVs were used at a m.o.i. of 10, except VV-MDR1(T7) and VV-T7, which were used at a m.o.i. of 250. *B*, the m.o.i. of rVVs expressing P-glycoprotein and T7 are indicated; other rVVs were used at a m.o.i. of 10.

peptides to K^d, we used T_{CD8+} specific for undefined VV peptides or for the 147–155 peptide from influenza virus NP. We previously showed that $NP_{147-155}$-specific T_{CD8+} are capable of lysing mouse cells expressing less than 30 complexes per cell (28).

As reported (13), lysis of rVV-infected T2 cells by VV-specific T_{CD8+} requires the expression of TAP[1 + 2] (Fig. 3). Expression of P-glycoprotein from the p7.5 promoter failed to sensitize cells for lysis above background values. Higher levels of P-glycoprotein expression, however, driven by the T7 polymerase, resulting in the generation of sufficient class I-peptide complexes to enable T_{CD8+}-mediated lysis.

In the experiment shown in Fig. 3*A*, we infected cells with VV-T7 and VV-MDR1(T7) at a m.o.i. of 250, a high dose in this system. The effect of m.o.i. of VV-MDR(T7) on the rescue of antigen presentation was further examined in an additional experiment in which cells were infected with VV-T7 and VV-MDR1(T7) at a m.o.i. of 10 (Fig. 3*B*) or 200 (Fig. 3*C*). This demonstrated that presentation of the VV-derived peptide required a high m.o.i. of these two rVVs. Importantly, this experiment also revealed that antigen presentation was rescued to a similar extent by infection with VV-CM, which expresses P-glycoprotein with no detectable function.

In the next experiment, we coinfected cells with VV-MDR1(T7) and a rVV encoding a cytosolic peptide corresponding to influenza virus $NP_{147-155}$ (with an initiating Met). We have shown that TAP-expressing cells infected with the rVV produce enormous numbers of the peptide-K^d complex (~50,000 copies/cell; Ref. 28). In conjunction with polyclonal NP-specific T_{CD8+} that are capable of lysing cells expressing few K^d-$NP_{147-155}$ complexes at the cell surface, this system should provide a sensitive measure of P-glycoprotein mediated transport of cytosolic peptide. As reported previously (13), presentation of $NP_{147-155}$ is completely TAP-dependent (Fig. 4, *top*). Using this sensitive system, infection with VV-MDR1(T7) at a m.o.i. of 20 was able to produce sufficient numbers of complexes to enable T_{CD8+} lysis of some cells. This presentation was only slightly affected by verapamil, however, which affected TAP-mediated presentation to a similar extent and is, therefore, probably acting in a nonspecific manner.

We next examined whether P-glycoprotein can specifically complement TAP1 or TAP2 subunits expressed individually. As seen in Fig. 4, *bottom,* coexpression of TAP1 or TAP2 with P-glycoprotein in T2 cells had no effect on the presentation of K^d-restricted, VV-derived determinants as compared to cells infected with a control rVV (VV-$NP_{147-155}$). In the same experiment, coinfection with VV-TAP[1 + 2] resulted in an easily detected increase in antigen presentation.

Taken together, these experiments indicate that VV-expressed P-glycoprotein is unable to specifically transport cytosolic peptides to

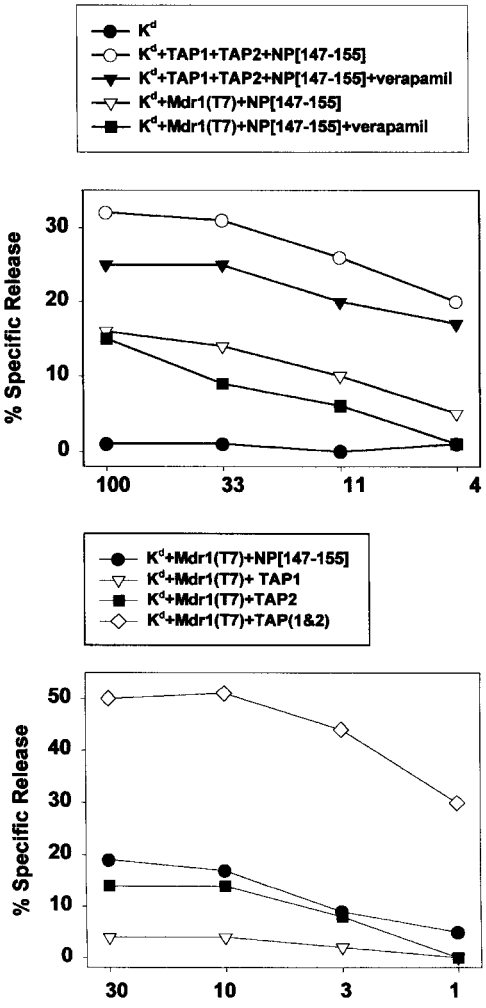

Fig. 4. Effect of verapamil on antigen presentation in rescued T2 cells. T2 cells infected for 4 h with the rVVs as indicated at a m.o.i. of 20 in the absence or presence of verapamil (10 μM) were tested in a 6-h cytotoxicity assay using secondary *in vitro* restimulated $NP_{147-155}$ (*top*) or VV-specific (*bottom*) effector cells at the effector to target ratio indicated. When used, verapamil was maintained throughout all incubation steps and the ^{51}Cr release assay.

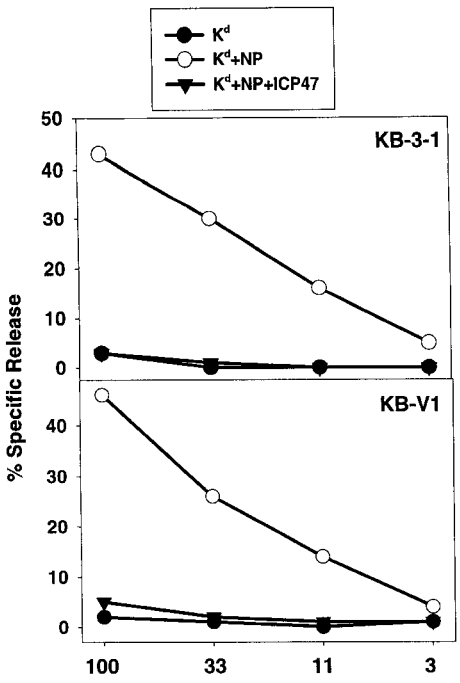

Fig. 5. Inhibitory effect of ICP47 on antigen presentation in KB-3-1 and KB-V1 cells. KB-3-1 and KB-V1 cells were infected with the indicated rVVs at m.o.i. of 10 for 4 h before testing in a 6-h cytotoxicity assay using secondary *in vitro* restimulated $NP_{147-155}$-specific effector cells at the indicated effector to target ratio.

MHC class I molecules, but that it can enable peptide delivery by nonspecific effects, possibly by disrupting membrane integrity.

Effect of P-Glycoprotein Expression on Presentation of Cytosolic Peptides by KB Cells. To examine the possible contribution of P-glycoprotein or other proteins present in drug-selected cells to the generation of MHC class I complexes, we examined the ability of ICP47 to block presentation of $NP_{147-155}$ from full-length NP in vinblastine-selected and nonselected KB cells. Cells were coinfected with VV-K^d, VV-NP, and either VV-ICP47 or a control rVV and tested for lysis by $NP_{147-155}$ specific T_{CD8+}. As seen in Fig. 5, presentation of NP was completely blocked by ICP47 in both selected and nonselected cells. Taken together, our data do not support a role of P-glycoprotein or other proteins that may contribute to the MDR phenotype in the delivery of ligands to MHC class I molecules.

DISCUSSION

In this study, we have examined the ability of P-glycoprotein to contribute to MHC class I biogenesis by measuring the ability of P-glycoprotein to: (*a*) deliver synthetic peptides to the ER of permeabilized TAP-deficient cells, as determined by peptide glycosylation; (*b*) enhance class I cell surface expression on TAP-deficient cells; and (*c*) enable TAP-independent presentation of cytosolic antigenic peptides. We will discuss the results of these approaches individually.

Peptides were not detectably transported into the ER despite the high levels of synthesis of P-glycoprotein in T2 cells infected with VV-MDR(T7). Because the half-time for P-glycoprotein export from the ER is ~1 h (29), very high levels of P-glycoprotein should be present in the ER of these cells, making the lack of peptide transport all the more compelling. Although we cannot eliminate the possibility that the lack of peptide transport is due to nonspecific effects of VV infection on cellular metabolism or P-glycoprotein function, this is unlikely, because we also failed to detect TAP-independent peptide transport in KB-V1 cells using a synthetic peptide corresponding to $ICP47_{1-35}$ to inhibit TAP.

There are two alternative explanations for the lack of detectable P-glycoprotein-mediated peptide transport. First, if P-glycoprotein is active in the ER, it may not be able to transport the indicator peptides used. Virtually all defined P-glycoprotein substrates are highly hydrophobic. The drug-binding sites on P-glycoprotein reside within the transmembrane domain (30, 31), and it is believed that P-glycoprotein generally recognizes substrates within the lipid bilayer, transporting molecules that intercalate into the membrane (31, 32). In contrast, TAP is thought to acquire peptides from the cytosol, and such peptides are far more hydrophilic than P-glycoprotein substrates. Indeed, antigenic peptides of sufficient hydrophobicity to access P-glycoprotein may not even require transport into the ER. This may account for the TAP-independent presentation of hydrophobic peptides present in leader sequences (33, 34).

In addition to problems of peptide access to P-glycoprotein, antigenic peptides, which are at least 8 residues in length, may be too long to be transported by P-glycoprotein. Sharom *et al.* (7) studied the interaction a series of synthetic hydrophobic peptides (N-acetyl-Leu_n-Tyr-amide) with P-glycoprotein. The interaction was maximal for the tripeptide and decreased with increasing peptide length, so that even the hexapeptide had little effect on drug transport.

Second, it is uncertain whether nascent P-glycoprotein is active in the ER. Although it is possible that P-glycoprotein is functional intracellularly, the location of such active P-glycoprotein has not been established, and it is possible that activity is gained only upon export from the Golgi complex. Because N-linked glycosylation only occurs in the ER, we cannot determine whether the reporter peptides were transported across plasma or Golgi complex membranes. Because both the cell surface and Golgi complex possess peptide-receptive class I molecules (27, 35), this could still enable P-glycoprotein to provide peptides to class I molecules.

Our failure to detect specific P-glycoprotein enhancement of class I expression (or antigen presentation) argues, however, against the possibility that cell surface or Golgi complex P-glycoprotein can transport class I binding peptides. These findings conflict with those of Masci *et al.* (10), who reported that the MDR phenotype was associated with enhanced binding of the W6/32 mAb to the cell surface. Possibly, this is due to distinct behaviors exhibited by different class I allomorphs expressed by KB and T2 cells *versus* the various cells used by Masci *et al.* (10). Although Masci *et al.* (10) demonstrated that the effect of P-glycoprotein on class I expression occurred posttranslationally, they did not distinguish whether the enhanced binding of W6/32 (and four other class I specific mAbs) was due to the production of more stable class I molecules due to an increase in the supply of high-affinity peptides (or the efficiency of loading such peptides in the ER) *versus* the reduced destruction of class I molecules once they are exported from the ER. The latter could occur as a result of P-glycoprotein-induced alterations in cellular physiology that affect the stability of class I molecules in transit or once they have arrived at the cell surface. Drug selection has been reported to be associated with intracellular alkalization, membrane depolarization, and other cellular alterations, any of which might affect the stability of class I molecules (36).

Masci *et al.* (10) also reported that incubation of PBLs with verapamil for 12 h decreased the binding of W6/32 by 54–60% and concluded that P-glycoprotein also plays a major role in the delivery of antigenic peptides to class I molecules in PBLs. Several findings are difficult to reconcile with this interpretation. First, PBLs in TAP −/− mice demonstrate a 90% decrease in the expression of cell surface class I molecules (37). Second, such decreases in the cell surface expression of W6/32 reactive class I molecules are difficult to achieve even when cells are treated with brefeldin A, which completely blocks transport of new molecules to the cell surface. Rather,

it seems likely that the effect of verapamil on class I expression in PBLs results from general effects on exocytosis, membrane protein turnover, or a combination of these factors.

In contrast to the inability of P-glycoprotein to mediate peptide translocation into the ER or enhance class I cell surface expression, we did find that VV-mediated P-glycoprotein expression enabled the presentation of TAP-dependent peptides to virus-specific T_{CD8+}. As T_{CD8+} lysis is the most sensitive method of measuring an effect of P-glycoprotein on antigen presentation, we carefully analyzed whether this small effect, observed only at extreme conditions of overexpression of peptide or P-glycoprotein truly reflected P-glycoprotein-mediated transport of cytosolic peptides. Our findings strongly suggest that the effect of P-glycoprotein was nonspecific, in the sense that it did not occur via the mechanism used by P-glycoprotein to translocate substrates across membranes. Thus, presentation of $NP_{147-155}$ was not significantly diminished by verapamil, a well-characterized P-glycoprotein inhibitor, and cells expressing VV-encoded functionally defective ATP-site mutants of P-glycoprotein presented peptides no worse than cells expressing functional P-glycoprotein. The ability of ICP47 to completely block presentation of $NP_{147-155}$ in KB-V1 cells is completely consistent with this conclusion and suggests that the nonspecific effect of P-glycoprotein on antigen presentation we observe is not biologically significant.

In summary, our findings strongly suggest that P-glycoprotein is not responsible for the presentation of cytosolic antigens in TAP-deficient cells, and is not a significant contributor of antigenic peptides, even when it is overexpressed in multidrug-resistant cells.

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