Multiple Antigen-Specific Processing Pathways for Activating Naive CD8⁺ T Cells In Vivo

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Multiple Antigen-Specific Processing Pathways for Activating Naive CD8+ T Cells In Vivo

Christopher C. Norbury,* Michael F. Princiotta,* Igor Bacik,* Randy R. Brutkiewicz,2
Philip Wood,† Tim Elliott,† Jack R. Bennink,3* and Jonathan W. Yewdell3*

Current knowledge of the processing of viral Ags into MHC class I-associated ligands is based almost completely on in vitro studies using nonprofessional APCs (pAPCs). This is two steps removed from real immune responses to pathogens and vaccines, in which pAPCs activate naive CD8+ T cells in vivo. Rational vaccine design requires answers to numerous questions surrounding the function of pAPCs in vivo, including their abilities to process and present peptides derived from endogenous and exogenous viral Ags. In the present study, we characterize the in vivo dependence of Ag presentation on the expression of TAP by testing the immunogenicity of model Ags synthesized by recombinant vaccinia viruses in TAP1−/− mice. We show that the efficiency of TAP-independent presentation in vitro correlates with TAP-independent activation of naive T cells in vivo and provide the first in vivo evidence for proteolytic processing of antigenic peptides in the secretory pathway. There was, however, a clear exception to this correlation; although the presentation of the minimal SIINFEKL determinant from chicken egg OVA in vitro was strictly TAP dependent, it was presented in a TAP-independent manner in vivo. In vivo presentation of the same peptide from a fusion protein retained its TAP dependence. These results show that determinant-specific processing pathways exist in vivo for the generation of antiviral T cell responses. We present additional findings that point to cross-priming as the likely mechanism for these protein-specific differences. The Journal of Immunology, 2001, 166: 4355–4362.

CD8+ T cells (TCD8+) recognize MHC class I molecules bearing oligopeptides derived from viral or self proteins (1, 2). Viral peptides (or their precursors) are usually generated in the cytosol of virus-infected cells by the action of proteasomes (3, 4). Recent evidence suggests that the major substrates of proteasomes are short lived defective ribosomal products that never reach a folded conformation after their synthesis (5). Cytosolic peptides of between 8 and 16 residues are transported into the endoplasmic reticulum (ER)4 of cells by TAP (6–8). TAP consists of two subunits, TAP1 and TAP2, both of which are necessary for TAP to function (reviewed in Ref. 9). In cells lacking TAP, the number of peptide-binding class I molecules expressed on the cell surface is reduced ~10-fold (10).

TAP is not the sole mechanism for potential class I ligands to gain entry to the ER. Peptides within ER targeting sequences of secreted or membrane proteins can be presented relatively efficiently by TAP-deficient cells (11–13). More generally, class I-binding peptides immediately COOH terminal to ER targeting sequences are efficiently presented in a TAP-independent manner (11, 14), and such minigene products are highly immunogenic in vivo (15, 16). Positioning peptides at the COOH terminus of secreted or type II membrane-anchored proteins also enables their TAP-independent presentation (termed the C-end rule), but usually at much lower efficiencies in the absence of a specific ER-associated proteolytic cleavage event (17). More sporadically, peptides can be liberated from internal sequences of ER-targeted proteins, and in some cases this can be influenced by N-linked glycosylation (18). The contribution of proteases in the secretory compartment to Ag presentation is based entirely on in vitro studies, a situation that we rectify in this report.

It is generally assumed that viral Ags are presented to naive TCD8+ in vivo by professional APCs (pAPCs). An important question is under what circumstances viral Ags are presented by direct priming (infected pAPCs presenting endogenous viral gene products) vs cross-priming (uninfected pAPCs presenting gene products synthesized by another host cell) (19). In the event of cross-priming, the rules governing TAP dependence of presentation of Ag in vitro may be discarded in vivo. The rules governing the priming of naive TCD8+ are currently based on in vitro studies of Ag presentation. In the present study, we use TAP−/− mice to gain insight into the mechanisms of Ag presentation to naive TCD8+.

**Materials and Methods**

**Mice**

TAPI−/− mice (10), a generous gift from Dr. Luc Van Kaer (Vanderbilt University School of Medicine, Nashville, TN) were bred onto a B6 background. OT-1 mice (20) were a generous gift from Dr Kristin Hogquist (University of Minnesota). C57BL/6, B6 TAPI−/−, OT-1, and F5 (21) mice were all bred at Taconic Farms (Germantown, NY). Bm1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

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Recombinant vaccinia viruses (rVV)

rVVs expressing antigenic peptides from vesicular stomatitis virus (VSV) nucleocapsid (N), Sendai virus nucleoprotein (NP), OVA, and influenza NP as outlined in Table I have been previously described. PR8 NP was directed to the secretory pathway using the signal sequence from IFN-β (22). Minigen constructs, with the exception of NT60 NP constructs, were targeted to the secretory pathway using the signal sequence from the adenovirus E3/19K protein (14). All NT60 NP constructs were targeted to the ER using the signal sequence from the influenza hemagglutinin (HA) protein (23).

rVVs expressing either human TAP1 or human TAP2, or both, have been described (24), as have double recombinants encoding both S-ova257-264 plus β2-microglobulin (β2m) and K b plus β2m (25). S represents a signal leader sequence that targets the peptide to the ER. The full-length murine TAP1 was inserted into the thymidine kinase locus of the rVV. S-nt60 plasmid to express foreign proteins under the control of the VV IE1 promoter. Expression of H2-K b and D b molecules when examined by cytofluorography using conformation-specific Abs (Y3 and B22, respectively). The rVVs expressing antigenic peptides at 1 μg/ml. After culture, live cells were recovered via a Ficoll-Hypaque gradient. The radioactivity in supernatants collected after 4–6 h incubation at 37°C was determined using a gamma counter. The percent specific release was then determined as: % specific release = [(CTL-induced release − spontaneous release)/release by detergent − spontaneous release]] × 100.

Purification and adoptive transfer of TCR-transgenic (Tg) T cells

Two days before transfer of TCR Tg T cells, recipient mice were irradiated with 800 rad. T cell-enriched populations were obtained from TCR Tg mice as follows. Lymph nodes (popliteal, inguinal, brachial, axillary, and superficial cervical) and spleen were taken and a single-cell suspension of mice was generated. Live cells were isolated via a Ficoll-Hypaque gradient and cells were incubated with anti-CD8 microbeads (Miltenyi Biotech, Auburn, CA) for 20 min before isolation over R5-positive selection columns held in an OctoMacs apparatus. After removal from the magnet, cells were eluted to give a cell population that was 70–90% CD8+.

Splenocytes (from C57BL/6 mice) or purified T CD8+ T cells (from TAP1−/− mice) were used as effectors in microcytotoxicity assays. Generally, 104 target cells were labeled with 100 μCi Na235CrO4 (Amer sham, Arlington Heights, IL) in a minimum volume of medium at 37°C for 60 min. When the activity of effectors from TAP1−/− mice was assayed, TAP2−/− RMA/S cells, pulsed with a specific or irrelevant K b or D b binding peptide, were used as targets. After two washes, 105 cells were aliquoted into round-bottom 96-well plates containing serial dilutions of effector TCD8+. The radioactivity in supernatants collected after 4–6 h incubation at 37°C was determined using a gamma counter. The percent specific release was then determined as: % specific release = [(CTL-induced release − spontaneous release)/release by detergent − spontaneous release] × 100.

Table I. Description of rVV used

<table>
<thead>
<tr>
<th>Antigenic Peptide</th>
<th>rVV Name</th>
<th>Protein Expressed</th>
<th>Requirement for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TAP in vitro</td>
</tr>
<tr>
<td>RGYYVQGL</td>
<td>VSV N</td>
<td>VSV nucleocapsid</td>
<td>+</td>
</tr>
<tr>
<td>RGYYVQGL</td>
<td>VSV N2−59</td>
<td>VSV nucleocapsid</td>
<td>–</td>
</tr>
<tr>
<td>RGYYVQGL</td>
<td>S-VSV N2−59</td>
<td>ER-targeted VSV nucleocapsid</td>
<td>–</td>
</tr>
<tr>
<td>RGYYVQGL</td>
<td>S-NP-VSV N2−59</td>
<td>ER-targeted influenza PR8 nucleocapsid C-terminal</td>
<td>–</td>
</tr>
<tr>
<td>SIINFEKL</td>
<td>OVA</td>
<td>OVA</td>
<td>–</td>
</tr>
<tr>
<td>SIINFEKL</td>
<td>OVA257-264</td>
<td>OVA peptide</td>
<td>–</td>
</tr>
<tr>
<td>SIINFEKL</td>
<td>S-OVA257-264</td>
<td>ER-targeted OVA peptide</td>
<td>–</td>
</tr>
<tr>
<td>SIINFEKL</td>
<td>NP-OVA257-264</td>
<td>PR8 nucleoprotein C-terminal OVA peptide</td>
<td>+</td>
</tr>
<tr>
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<td>S-NP-OVA257-264</td>
<td>ER-targeted PR8 nucleoprotein C-terminal OVA peptide</td>
<td>–</td>
</tr>
<tr>
<td>FAPGNYPAL</td>
<td>SEN NP24−32</td>
<td>Sendai virus nucleocapsid peptide</td>
<td>–</td>
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<td>ER-targeted Sendai virus nucleocapsid peptide</td>
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<td>ASNEMNADAM</td>
<td>NT60 NP</td>
<td>Influenza NT60 nucleoprotein</td>
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<td>NT60 NP366−374</td>
<td>NT60 nucleoprotein peptide</td>
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<td>ER-targeted NT60 nucleoprotein (364–374)</td>
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<tr>
<td>ASNEMNADAM</td>
<td>S-NT60 NP328−498</td>
<td>ER-targeted NT60 nucleoprotein (1, 3, 282–498)</td>
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<tr>
<td>SIINFEKL</td>
<td>S-OVA257-264 + β2m</td>
<td>ER-targeted OVA peptide and mouse β2m</td>
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<tr>
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<td>ER-targeted influenza (PR8) nucleoprotein peptide</td>
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<td>ASNENMETM</td>
<td>S-NP566−374</td>
<td>ER-targeted influenza (PR8) nucleoprotein peptide</td>
<td>NA</td>
</tr>
</tbody>
</table>

* NA, Not applicable.
via the tail vein. Two hours after injection of T cells, mice were immunized with 5 × 10^5 PFU rVV i.v. Two days later, spleens were removed and analyzed as outlined below.

Flow cytometric analysis

For analysis of cell division in vivo, spleens were harvested from two mice per group and homogenized, and the cells were pooled. Mononuclear cells were isolated by centrifuging over lymphocyte separation medium (Bio-Whittaker, Walkersville, MD) and harvesting the cells at the lymphocyte separation medium-medium interface. Cells were incubated in 2,4-DG supernatant, 20% normal mouse serum for 20 min on ice to block Fc receptor-mediated uptake of Ab and then stained with either anti-Vα2-PE (clone B20.1; Pharmingen, San Diego, CA) or anti-Vβ11-PE (clone RR3-15; Pharmingen) Abs for 40 min on ice. Cells were washed five times; then data were captured using a FACSscan (Becton Dickinson, San Jose, CA). Only Vα2 (for OT-1)- or Vβ11 (for F5)-positive cells were analyzed for CFSE staining, and data were analyzed using FlowJo software (Tree Star, San Carlos, CA). Similarly, for analysis of cell surface staining of bone marrow-derived DCs, cells were harvested, FC receptors blocked, and then stained with FITC-conjugated 25.D1.16 (specific for H2-Kb-OVA257-264 complex) Ab at a 1:10 dilution for 30 min at 0°C before washing extensively in ice-cold PBS and analyzing as above.

Results

In vitro presentation of rVV gene products by TAP1+/− mouse cells

Our strategy to study in vivo processing of viral proteins entailed comparing the capacity of rVV expressing various forms of Ags to elicit TCD8 responses in TAP1+/− mice. To facilitate direct comparisons between presentation in vitro and in vivo, it was necessary to generate a permanent cell line from TAP1+/− mice. (The only TAP-deficient mouse cell line available, RMA/S, lacks TAP2 and has a number of additional mutations resulting from the chemical mutagen used for its generation. RMA/S cells also suffer from being poorly infected by rVV virus.) By using SV40 (28) to transform kidney cells ex vivo, we cloned a TAP1+/− cell line (1E12 cells). 1E12 cells were infected with rVV encoding a number of well-characterized H2-Dk-restricted antigenic peptides in different protein contexts (Table I). Infected cells were examined for recognition by Tcells specific for the corresponding determinant using a standard 51Cr microcytotoxicity assay.

These findings are summarized in Table I because they largely mirror previous in vitro findings with the same rVVs used in human TAP-deficient cells (29, 30). As in prior studies (14), the highest levels of lysis were achieved by infecting cells with an ER-targeted minigene. Expressing the full-length gene product that is the source of the peptide resulted in the lowest levels of lysis (usually slightly above or at levels of lysis observed with control rVVs). Infection with rVVs expressing the peptide at the COOH terminus of a secreted protein resulted in intermediate levels of lysis, as did targeting influenza NP residues 328–498 to the ER using the leader sequence of influenza HA.

Unexpectedly, three of the four peptides tested (all but NP 366-374) were presented at intermediate levels as cytosolic minigenes (scored as TAP independent in Table I). Given the vast number of peptides that are usually produced from cytosolic minigenes (25, 31), we attribute the modest levels of presentation to “leakiness,” i.e., a low efficiency TAP-independent alternate means of entering the ER. (Although it is formally possible that TAP2 forms a low efficiency channel, we have never seen evidence supporting this in comparison presentation of TAP1 and TAP2-deficient cells with TAP2-expressing cells.)

The cytotoxicity assay is an extremely blunt tool for quantitating levels of peptide-class I complexes; once the threshold number of complexes is reached on a given cell for triggering TCD8 lysis, further increases are not registered by the assay (a cell can die but once). To more accurately quantify complex expression, we examined the increase in expression of conformed class I molecules on the surface of TAP1−/− cells after infection with rVVs by staining with the conformation-dependent Abs to H2-Kb (Y3) or H2-Dk (B22). We were unable to detect increases in the expression of class I molecules on 1E12 cells after infection with rVVs encoding either TAP or appropriate ER-targeted peptides, probably owing to relatively low levels of rVV gene expression in combination with low levels of class I synthesis (not shown).

We therefore turned to DC prepared from the bone marrow of TAP1−/− mice by short term culture in GM-CSF-containing medium. Infection of TAP1−/− DC with rVVs expressing either mouse or human TAP1 resulted in a slight (1.5-fold) but significant increase in Kb cell surface expression (not shown). Curiously, infection with rVV expressing both human TAP subunits resulted in a greater enhancement of Kb expression (3-fold). A similar enhancement was observed after infection with ER-targeted OVA257-264 (VV-S-OVA257-264). To test whether this up-regulation of Kb expression on the cell surface was due to stabilization by the OVA257-264 peptide, cells were stained with an Ab specific for the H2-Kb-OVA257-264 complex (25). As shown in Fig. 1, infection with ER-targeted OVA257-264 (VV-S-OVA257-264) induced an approximate 3-fold up-regulation of Kb-OVA257-264 complexes on the cell surface. By contrast, neither Kb expression nor expression of the specific Kb-OVA257-264 complex was changed after infection with rVVs expressing full-length OVA (VV-OVA), cytosolic minimal peptide determinant (VV-OVA257-264), or the minimal peptide determinant OVA257-264 attached to the COOH terminus of ER-targeted influenza NP (VV-S-NP-OVA257-264). This confirms that ER-targeting results in the generation of far greater numbers of Kb-OVA257-264 complexes in TAP1−/− cells than are generated from other constructs.

Primining of a CTL response in TAP1−/− mice

We next turned our attention to the main question: how does in vitro Ag presentation compare with in vivo CTL priming? We infected TAP1−/− mice with the rVVs described above and measured lytic activities after secondary stimulation of splenocytes in

Flourescence (No. of OVA257-264 H2-Kb complexes)

FIGURE 1. Ag presentation by TAP1−/− DC. TAP1−/− bone marrow-derived DC were infected with recombinant rVV expressing the OVA257-264 determinant in various contexts for 6 h and then stained with the FITC-conjugated 25.D1.16 Ab specific for the OVA257-264-H2-Kb complex. Bone marrow DC were infected with VV-S-NP60 NP266-374, VV-S-OVA257-264, VV-OVA, VV-S- or NP-OVA257-264. Hatched lines represent staining with the irrelevant control (Con) (S-NP147-155) for comparison.
vitro with the appropriate synthetic peptide. TAP1−/− mice possess only 10% as many TCD8+ as in normal mice (10). Despite this, Sandberg et al. (32) have shown that TAP1−/− mice maintain the ability to respond to selected determinants after immunization with synthetic peptides in adjuvant, including VSV N52–59 and SEN NP324–332, but not NT60 NP366–374 or OVA257–264. Indeed, after infection with rVVs expressing the corresponding ER-targeted versions of these peptides, TCD8+ responses to VSV N52–59 and NP324–332 were easily detected (Fig. 2). Like Sandberg et al., we did not detect responses to S-OVA257–264 or NT60 NP366–374 (not shown), supporting the conclusion that appropriate TCD8+ are missing from the repertoire of TAP1−/− mice. In the same experiment, TAP1−/− mice failed to respond to rVVs encoding full length VSV N, cytosolic minigene versions of VSV N52–59, SEN NP324–332 (not shown), or the same peptides appended to the COOH terminus of ER-targeted NP. Taken together, the present (Fig. 1) and previous results (11, 14) indicate that peptide class I complexes are generated most efficiently in TAP deficient cells by expressing ER-targeted peptides. These results indicate that TAP−/− mice were able to respond only to rVV-infected cells that express peptide-class I complexes at the highest densities on the cell surface of TAP-deficient cells.

**TAP dependence of CTL priming after reconstitution of TAPI−/− mice with NP366–374-specific TCR Tg TCD8+**

We bypassed difficulties with limitations in the TCR repertoire of TAPI−/− mice by reconstituting the mice with purified TCD8+ from TCR Tg mice. To generalize the results, we used two strains of TCR Tg mice; F5, which generate TCD8+ specific for Db-NT60 NP366–374 complexes, and OT-1, which generate TCD8+ specific for Kb-OVA257–264 complexes. TAPI−/− mice generate strong and rapid cytolytic responses against adoptively transferred TCD8+ expressing normal levels of cell surface class I molecules (reviewed in Ref. 33). To avoid rejection of adoptively transferred Tg TCD8+ recipients were irradiated with 800 rad 2 days before transfer. TCD8+ were labeled with CFSE just before injection to enable flow cytometric determination of cell division, and mice were infected with rVV-S 2–3 h later. Similar results were obtained by measuring ex vivo cytotoxicity, but this required greater numbers of animals and was much more variable than CFSE staining, which became the method of choice.

Reconstitution of TAPI−/− mice with F5 TCR Tg TCD8+, followed by infection with VV-S-NT60 NP366–374 caused significant proliferation of the F5 TCD8+ 2 days postinfection (Fig. 3). Proliferation was undetected 1 day postinfection under these conditions (not shown), or on day 2 if the mice were uninfected. The specificity of activation of the F5 cells was best illustrated by their failure to proliferate after infection with an rVV expressing an ER-targeted version of an homologous peptide from the PR8 NP that possesses two amino acid substitutions. Despite binding to Dα molecules with high affinity, this peptide is not recognized by F5 cells in vitro, even at very high concentrations (21). In the same experiment, we found that F5 cells proliferated after infection with VV-S-NT60 NP328–498, but not rVV expressing the cytosolic peptide NP366–374 (VV-NT60 NP366–374), normal NP (VV-NT60 NP), or ER-targeted NP (VV-S-NT60 NP) (Fig. 3). Therefore, in this system, in vivo priming closely parallels in vitro presentation.

Although it is formally possible that we are simply infecting the transferred transgenic TCD8+, which then serve as TAP-expressing APCs, this is extremely unlikely. First, we injected highly purified TCD8+. These express vaccinia proteins at low levels, if at all, because mouse T cells are highly resistant to infection with VV. Second, and more directly, the cytosolic minigene, which is presented at higher levels in vitro than VV-S-NT60 NP328–498, failed to activate transferred F5 cells. Thus, the evidence strongly supports the conclusion that TAP-independent presentation occurs with minigene or longer NP constructs that are targeted to the ER, providing the initial in vivo demonstration of processing of class I peptides in the secretory pathway.

**TAP dependence of CTL priming after reconstitution of TAPI−/− mice with OVA257–264-specific TCR Tg TCD8+**

We expanded these findings using the same experimental protocol to examine the presentation of Ags to OT-1 TCD8+, TAPI−/− mice reconstituted with CFSE-labeled OT-1 TCD8+ displayed a strong proliferative response 2 days postinfection with VV-S-OVA257–264, whereas no proliferation was observed if mice were uninfected (data not shown) or infected with a control rVV encoding S-NP147–155 (Fig. 4). OT-1 TCD8+ also proliferated after infection with VV-S-NP-OVA257–264, providing evidence for the in vivo relevance of the C-end rule (17). The degree of proliferation was lower than that after VV-S-OVA257–264 infection, however, demonstrating that OT-1 proliferation is not all or none but rather is related to the number of Kb-OVA257–264 complexes generated by APCs. In contrast to the findings with the F5 system, infection with rVV expressing the cytosolic minimal determinant triggered OT-1 proliferation in TAPI−/− mice, in this case to a degree similar to that of VV-S-NP-OVA257–264. The in vivo presentation of OVA257–264 and NT60 NP366–374 minimal determinant products in TAPI−/− mice parallels their in vitro presentation in TAPI−/− cells (Table I).

CFSE-labeled OT-1 cells proliferated after infection with VV-OVA. The magnitude of the response was much lower than that seen with even VV-OVA257–264, but some cells had divided three times in response to VV-OVA. This was confirmed by ex vivo cytotoxicity assays performed 5 days following infection, in which VV-OVA induced OVA257–264 specific lysis above background values observed with uninfected mice or mice infected with a control rVV (not shown). By contrast, infection with rVV expressing
OVA at the COOH terminus of NP (which localizes to the cytosol/nucleus) failed to induce detectable proliferation of OT-1 TCR Tg CD8+ in vivo. Because VV-OVA and VV-NP-OVA are both completely TAP dependent in vitro, the difference in their in vivo presentation suggests that a different Ag processing pathway is used in vivo, possibly related to the nuclear cytosolic localization of NP-OVA257–264 vs the secretion of OVA.

Transferred TAP1+/−/− cells are not responsible for priming of a response to VV-OVA

As with the F5 system, our interpretation of the OT-1 results are entirely dependent on the conclusion that presentation is mediated by TAP1+/−/− APCs, and not cells transferred from OT-1 mice. To rigorously support this conclusion, we transferred CFSE-labeled OT-1 cells into Kbm1 mutant mice. Due to 3 amino acid substitutions in the α helix of the Kβ binding groove, these mice are incapable of presenting OVA 257–264 to OT-1 TCD8+. Thus, if priming were due to infection of the adoptively transferred OT-1 cells, then the cells should proliferate in Kbm1 mice after infection with the appropriate rVVVs. Although Kbm1 molecules present many of the same self peptides as wild-type Kβ, an allogeneic response is still mounted against transferred OT-1 cells; therefore, the same protocol as for TAP1+/−/− recipients was followed, with the Kbm1 mutant mice being irradiated 2 days before reconstitution with T cells.

Infection of Kbm1 mice with even the strongest stimulating virus in the OT-1 system, VV-S-OVA 257–264 , failed to stimulate proliferation of transferred OT-1 TCD8+. VV-OVA was also nonimmunogenic in Kbm1 mice. As a positive control, mice were infected with two rVVVs, one expressing wild-type Kβ and mouse β2 m (VV-Kβ1 β2 m), the other expressing S-OVA 257–264 and mouse β2 m (VV-S-OVA 257–264 + β2 m). Under these conditions, where cells doubly infected could express OVA 257–264 in complex with wild-type H-2Kβ, a strong proliferative response was observed (Fig. 5). Each of the rVVVs alone was nonimmunogenic under these conditions. These findings provide strong evidence that first priming in

Vivo presentation suggests that a different Ag processing pathway is used in vivo, possibly related to the nuclear cytosolic localization of NP-OVA257–264 vs the secretion of OVA.

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are capable of producing sufficient virus for priming, particularly in vivo and in vitro. After 3 h, mice were either infected with a recombinant rVV expressing full length OVA, left uninfected (negative), or immunized with spleen cells from normal or Kbm1 mice infected with a control VV virus (VV-S-NP147–155), OVA, or OVA257–264. Spleen cells were prepared by lysing RBC and infecting with 2 × 106 PFU virus per spleen for 1 h. After 6 h, TAP−/− mice reconstituted with OT-1 were immunized with ~3 × 106 cells from the infected spleen cells cultures i.v. B, Effect of the concentration of psoralen and time of exposure to UV irradiation on presentation of the OVA257–264 peptide in complex with H2-Kb on the surface of L-Kb cells. At the concentration and time used for inactivation of the virus in A (1 μg psoralen for 3 min), presentation of the peptide derived from cytosolic minigene is diminished but not ablated. At this concentration, presentation of peptide derived from full-length OVA is completely ablated, and no PFUs of virus can be detected.

**Diagram**

**FIGURE 6.** Cross-priming of rVV-encoded Ags in vivo. A, TAP1−/− mice were irradiated with 800 rad and 2 days later injected with 1 × 107 purified CFSE-labeled cells from an OT-1 TCR transgenic mouse. After 3 h, mice were either infected with a recombinant rVV expressing full length OVA, left uninfected (negative), or immunized with spleen cells from normal or Kbm1 mice infected with a control VV virus (VV-S-NP147–155), OVA, or OVA257–264. Spleen cells were prepared by lysing RBC and infecting with 2 × 106 PFU virus per spleen for 1 h. After 6 h, TAP1−/− mice reconstituted with OT-1 were immunized with ~3 × 106 cells from the infected spleen cells cultures i.v. B, Effect of the concentration of psoralen and time of exposure to UV irradiation on presentation of the OVA257–264 peptide in complex with H2-Kb on the surface of L-Kb cells. At the concentration and time used for inactivation of the virus in A (1 μg psoralen for 3 min), presentation of the peptide derived from cytosolic minigene is diminished but not ablated. At this concentration, presentation of peptide derived from full-length OVA is completely ablated, and no PFUs of virus can be detected.

**Discussion**

In this paper, we show that in general there is a good correlation between the TAP dependence of the in vitro generation of class I-peptide complexes and in vivo activation of naive TCD8+. Probably the most interesting finding we uncovered is an exception to this rule, that there is a clear-cut difference between the TAP dependence of in vitro and in vivo presentation of VV-expressed OVA, the former being TAP dependent, the latter TAP independent. This discrepancy points to a qualitative difference in the routes of Ag processing used. The observation that TCD8+ are activated in TAP−/− mice by immunization with VV-OVA-infected Kbm1 cells strongly suggests that VV-OVA priming in TAP−/− mice is based on a cross-priming mechanism.

What special properties of OVA might account for its cross-priming properties? An important clue comes from the inability of VV-NP-OVA257–264 to activate OVA257–264-specific TCD8+ in TAP−/− mice. In TAP-expressing cells, OVA257–264 is presented at similar efficiencies from NP-OVA257–264 vs OVA, suggesting that the enhanced TAP-independent immunogenicity of OVA is unlikely to reflect increased levels of cytosolic chaperones bearing OVA257–264-containing peptides. This shifts suspicion to the secretory nature of OVA, which unlike NP (or SEN-NP or VSV N, for that matter) is targeted to the secretory pathway, where it obtains an N-linked oligosaccharide and is efficiently secreted by cells. Although purified OVA is poorly immunogenic, perhaps the immunogenicity of biosynthesized OVA is enhanced by the local environment of a VV infection, or by molecular chaperones that remain associated with a minor fraction of newly secreted OVA.

The immunogenicity of OVA257–264 may be further enhanced in the context of OVA by the special properties of the OVA257–264 peptide and its immediate flanking residues. Perhaps it is representative of a subset of Ags that are able to withstand proteolysis in the endosomal compartment of pAPCs and associate with class I molecules that recycle to the cell surface for activating TCD8+.

**Figure 6**

**A**

**B**

**Legend:**

- **A**: Cross-priming of rVV-encoded Ags in vivo. A, TAP1−/− mice were irradiated with 800 rad and 2 days later injected with 1 × 107 purified CFSE-labeled cells from an OT-1 TCR transgenic mouse. After 3 h, mice were either infected with a recombinant rVV expressing full length OVA, left uninfected (negative), or immunized with spleen cells from normal or Kbm1 mice infected with a control VV virus (VV-S-NP147–155), OVA, or OVA257–264. Spleen cells were prepared by lysing RBC and infecting with 2 × 106 PFU virus per spleen for 1 h. After 6 h, TAP1−/− mice reconstituted with OT-1 were immunized with ~3 × 106 cells from the infected spleen cells cultures i.v. B, Effect of the concentration of psoralen and time of exposure to UV irradiation on presentation of the OVA257–264 peptide in complex with H2-Kb on the surface of L-Kb cells. At the concentration and time used for inactivation of the virus in A (1 μg psoralen for 3 min), presentation of the peptide derived from cytosolic minigene is diminished but not ablated. At this concentration, presentation of peptide derived from full-length OVA is completely ablated, and no PFUs of virus can be detected.

**Table**

<table>
<thead>
<tr>
<th>Fluorescence (CFSE Staining)</th>
<th>No. of Cells</th>
</tr>
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<tbody>
<tr>
<td>OVA</td>
<td>Negative</td>
</tr>
<tr>
<td>UV: OVA257–264</td>
<td>B6/OVA</td>
</tr>
<tr>
<td>B6/S-NP147–155</td>
<td>Bm1/OVA</td>
</tr>
<tr>
<td>Bm1/ OVA257–264</td>
<td>Bm1/OVA257–264</td>
</tr>
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**Discussion**

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The immunogenicity of OVA257–264 may be further enhanced in the context of OVA by the special properties of the OVA257–264 peptide and its immediate flanking residues. Perhaps it is representative of a subset of Ags that are able to withstand proteolysis in the endosomal compartment of pAPCs and associate with class I molecules that recycle to the cell surface for activating TCD8+.
We also provide the initial evidence that expression of a minimal Ag expressed in the cytosol is sufficient for cross-priming. Unlike the case with OVA, this probably occurs through binding of the peptide to either a cytosolic or ER molecular chaperone (37–39), because free peptides are degraded rapidly by cells (40). The recent finding that heat shock protein 70-mediated presentation of OVA\textsubscript{257–264} by pAPCs can be TAP independent (41) is consistent with this possibility.

In contrast to the present findings, it was previously reported that the presentation of VV-OVA to OVA\textsubscript{257–264}-specific T\textsubscript{CD8+} was completely TAP-dependent, inasmuch as the virus did not activate naïve T\textsubscript{CD8+} in irradiated B6 mice receiving bone marrow from TAP\textsuperscript{-/-} mice (42). In the same study, the presentation of VV-S-OVA\textsubscript{257–264} occurred in a TAP-independent manner, as we observed. We believe the discrepancy in the presentation of OVA is due to the lower sensitivity of the bone marrow chimeras relative to the transferred OT-1 T\textsubscript{CD8+}. Indeed, we demonstrate that VV-S-OVA\textsubscript{257–264} provides a stronger in vivo stimulus than OVA, and no doubt had we transferred less OT-1 cells we could have set the system to observe a positive response to VV-S-OVA\textsubscript{257–264} and negative response to VV-OVA. Similarly, our failure to demonstrate responses to various rVV in TAP\textsuperscript{1/-} mice cannot be interpreted as a total inability to present the corresponding Ags, but only as a failure to present Ag at the sensitivity of the method used for detection.

The second significant aspect of the present study is that it provides the initial in vivo demonstration of class I peptide processing in the secretory pathway. We detected TAP-independent presentation of a C-end rule determinant (S-NP-OVA\textsubscript{257–264}) as well as the endoproteolytic liberation of NP\textsubscript{366-374} from a secreted fragment of NP to adoptively transferred T\textsubscript{CD8+}. The parallels between in vivo and in vitro presentation of these Ags strongly suggest that their presentation occurs exclusively by direct presentation in TAP\textsuperscript{1/-} mice (and not cross-priming), because it is unlikely that the mechanisms governing the efficiency of TAP-independent cross-priming of various forms of Ag should directly parallel the mechanisms involved in entering the ER in a TAP-independent manner. Although the presentation of peptide can occur in the absence of TAP in vivo, it is possible that TAP may enhance the efficiency of this pathway, perhaps via stabilization of the MHC class I complex in the ER, allowing more peptide receptive MHC class I molecules to reach the secretory pathway.

Although this conclusion is limited to TAP-independent cross-priming, previous findings regarding the immunogenicity of VV suggest that TAP-dependent cross-priming of VV-encoded Ags may be the exception rather than the rule. It was shown a number of years ago that both influenza HA and NP inserted into VV elicit better mouse T\textsubscript{CD8+} response when controlled by an early promoter than by a late promoter, even when the late promoter constructs generate far greater quantities of the Ag (43). A study by Bronte et al. (44) provided a likely explanation for these findings: VV infection of mouse DC does not progress past the early phase of viral replication. This is consistent with the hypothesis that direct presentation accounts for most of the priming of rVV-encoded Ags, and, given that late viral proteins will still be produced in large quantities by other infected cells in the immunized animals, cross-priming does not detectably occur for many proteins.

In summary, the present findings support the idea that there are determinant-specific routes of presenting Ags in vivo to naive T\textsubscript{CD8+}. The routes used in any given circumstance will depend on multiple factors including the species immunized, route of immunization, dose of Ag, vector used or form of Ag administered, context of the immunogenic peptide, and the peptide itself. In combination, these factors will determine the extent to which direct priming vs cross-priming contributes to T\textsubscript{CD8+} activation. Cross-priming itself will no doubt also use a number of distinct mechanisms. We have provided the initial evidence that expression of a minimal Ag is sufficient for cross-priming. The most likely mechanism for this activity is the binding of the peptide to either a cytosolic or an ER molecular chaperone (37–39). We also note that cells expressing the cytosolic OVA\textsubscript{257–264} express far greater amounts of the peptide than cells expressing OVA. Therefore, it remains an open question to what extent cross-priming with OVA and other Ags is based on transfer of molecular chaperones bearing peptides as opposed to transfer of free forms of Ags, either full-length or truncated (45). Other crucial questions await answers including the nature of the pAPCs involved in direct and cross-priming and to what extent cross-priming Ags are acquired from live, necrotic, or apoptotic cells (46, 47).

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References


