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Multiple Antigen-Specific Processing Pathways for Activating Naive CD$^8^+$ T Cells In Vivo

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

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 CD$^8^+$ 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 TAP$^{-/-}$ 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.

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Materials and Methods

**Mice**

TAP$^{-/-}$ 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 TAP$^{-/-}$, 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).
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). Minigene 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 vaccinia virus (VV), removing a signal/leader sequence that targets the peptide to the ER. The full-length murine TAP1 was inserted into the thymidine kinase locus of vaccinia viruses (VVs) by homologous recombination as described using the pSC11 plasmid to express foreign proteins under the control of the VV early/late promoter (26). The coding sequence was verified by sequencing PCR-amplified copies of the full-length TAP1 gene isolated from the rVV.

Cell lines and cultures

All media were purchased from Life Technologies (Gaithersburg, MD). 1E12 cells were maintained in DMEM containing 10% FBS (D-10). CTL culture was in RPMI 1640 containing 10% FBS, 5 x 10^-5 M 2-mercaptoethanol, antibiotics (penicillin and streptomycin), nonessential amino acids, sodium pyruvate (1 mM), and 2 mM glutamine.

Bone marrow-derived dendritic cells (DC) were obtained as previously described (27). Briefly, bone marrow was flushed from femurs, and cells were resuspended in 20 ml D-10, to which penicillin and streptomycin, 2 mM glutamine.

Table I. Description of rVVs 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>
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<tr>
<td>RGYYQQGL</td>
<td>VSV N</td>
<td>VSV nucleocapsid</td>
<td>+</td>
</tr>
<tr>
<td>RGYYQQGL</td>
<td>VSV N22-59</td>
<td>VSV nucleocapsid peptide</td>
<td>-</td>
</tr>
<tr>
<td>RGYYQQGL</td>
<td>S-VSV N22-59</td>
<td>ER-targeted VSV nucleocapsid peptide</td>
<td>-</td>
</tr>
<tr>
<td>RGYYQQGL</td>
<td>S-NP-VSV N22-59</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>
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<tr>
<td>SIINFEKL</td>
<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 NP324-332</td>
<td>Sendai virus nucleocapsid peptide</td>
<td>+</td>
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<tr>
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<td>ER-targeted Sendai virus nucleocapsid peptide</td>
<td>-</td>
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<tr>
<td>FAPGNYPAL</td>
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<td>ER-targeted PR8 nucleoprotein with C-terminal</td>
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<td>ASNENMDAM</td>
<td>NT60 NP</td>
<td>Influenza NT60 nucleoprotein</td>
<td>+</td>
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<td>NT60 NP366-374</td>
<td>NT60 nucleoprotein peptide</td>
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<td>ER-targeted NT60 nucleoprotein peptide</td>
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<td>ER-targeted NT60 nucleoprotein (364-374)</td>
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<tr>
<td>ASNENMDAM</td>
<td>S-NT60 NP366-374</td>
<td>ER-targeted NT60 nucleoprotein (1, 2, 328-498)</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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<td>S-NP147-155</td>
<td>ER-targeted influenza (PR8) nucleoprotein peptide</td>
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<td></td>
<td>K2 + β2m</td>
<td>Mouse H-2 K2 and mouse β2m</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 \times 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 2.4G2 supernatant, 20% normal mouse serum for 20 min on ice to block Fc receptor-mediated uptake of Ab and then stained with either anti-Va2-PE (clone B20.1; PharMingen, San Diego, CA) or anti-Vb11-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 Va2 (for OT-1)- or Vb11 (for F5)-positive cells were analyzed for CFSE staining, and data were analyzed using FlowJo software (Treestar, 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-OVA 257–264 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 rVVs expressing various forms of Ags to elicit \( T_{CDR8} \) 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 H-2K\(^{d}\)-restricted antigenic peptides in different protein contexts (Table I). Infected cells were examined for recognition by \( T_{CDR8} \) 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 rVV). Infection with rVV 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 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 \( T_{CDR8} \) lysis, further increases are not registered by the assay (a cell can die but once). To more accurately quantitate 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-K\(^{b}\) (Y3) or H2-D\(^{b}\) (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 rVV expressing either mouse or human TAP1 resulted in a slight (1.5-fold) up-regulation of K\(^{b}\) cell surface expression (not shown). Curiously, infection with rVV expressing both human TAP subunits resulted in a greater enhancement of K\(^{b}\) 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 K\(^{b}\) expression on the cell surface was due to stabilization by the OVA257–264 peptide, cells were stained with an Ab specific for the H2-K\(^{b}\)-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 K\(^{b}\)-OVA257–264 complexes on the cell surface. By contrast, neither K\(^{d}\) expression nor expression of the specific K\(^{d}\)-OVA257–264 complex was changed after infection with rVV 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 K\(^{d}\)-OVA257–264 complexes in TAP1−/− cells than those generated from other constructs.

Fluorescence (No. of OVA257–264 H2-K\(^{b}\) complexes)

**FIGURE 1.** Ag presentation by TAP1−/− DC. TAP1−/− bone marrow-derived DC were infected with recombinant rVVs 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-K\(^{b}\) complex. Bone marrow DC were infected with VV-S-NF60 NP266–374, VV-S-OVA257–264, VV-OVA, VV-S, or NP-OVA257–264. Hashed 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 N\(_{52-59}\) and SEN NP\(_{324-332}\), but not NT60 NP\(_{366-374}\) or OVA\(_{257-264}\). Indeed, after infection with rVVs expressing the corresponding ER-targeted versions of these peptides, TCD8\(^+\) responses to VSV N\(_{52-59}\) and NP\(_{324-332}\) were easily detected (Fig. 2). Like Sandberg et al., we did not detect responses to S-OVA\(_{257-264}\) or NT60 NP\(_{366-374}\) (not shown), supporting the conclusion that appropriate TCD8\(^+\) are missing from the repertoire of TAP1\(^{-/-}\) mice. In the same experiment, TAPI\(^{-/-}\) mice failed to respond to rVVs encoding full length VSV N, cytosolic minigene versions of VSV N\(_{52-59}\), SEN NP\(_{324-332}\) (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 TAP1\(^{-/-}\) mice with NP\(_{366-374}\)-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 D\(^{b}\)-NT60 NP\(_{366-374}\) complexes, and OT-I, which generate TCD8\(^+\) specific for K\(^{a}\)-OVA\(_{257-264}\) 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.

We expanded these findings using the same experimental protocol to examine the presentation of Ags to OT-1 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 NP\(_{328-498}\), failed to activate transferred F5 cells. Thus, the evidence strongly supports the conclusion that TAP-independent presentation occurs with minigene and 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 OVA\(_{257-264}\)-specific TCR Tg TCD8\(^+\)**

![Graph](http://www.jimmunol.org/)

**FIGURE 2.** Priming of endogenous CD8\(^+\) T cells in TAPI\(^{-/-}\) mice. TAPI\(^{-/-}\) mice were infected with VV-S VSV N\(_{52-59}\), VV-VSV N, VV-S-VP VSV N\(_{52-59}\), VV-S-VP VSV N\(_{52-59}\), VV-S-NP VSV N\(_{52-59}\), or VV-S-NP SEN NP\(_{324-332}\). Three weeks after infection, spleens were removed and restimulated with specific peptide in vitro. After 5 days, cytotoxicity was assayed in a conventional chromium release assay against RMA-S cells pulsed with a specific or irrelevant peptide (OVA\(_{257-264}\)). Results are expressed as percentage of maximal lysis.

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-OVA\(_{257-264}\) but some cells had divided three times in response to VV-OVA. This was confirmed by ex vivo cytotoxicity assays performed 5 day following infection, in which VV-OVA induced OVA\(_{257-264}\) 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$_{257-264}$ at the COOH terminus of NP (which localizes to the cytosol/nucleus) failed to induce detectable proliferation of OT-1 T$_{CRD8}^1$ in vivo. Because VV-OVA and VV-NP-OVA$_{257-264}$ 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-OVA$_{257-264}$ vs the secretion of OVA.

**Transferred TAP$^{1/-}$ 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 TAP$^{1/-}$ 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 $\alpha$ helix of the K$^b$ binding groove, these mice are incapable of presenting OVA$_{257-264}$ to OT-1 T$_{CRD8}^1$ cells (34). 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 rVVs. Although Kbm1 molecules present many of the same self peptides as wild-type K$^b$, an allogeneic response is still mounted against transferred OT-1 cells; therefore, the same protocol as for TAP$^{1/-}$ 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 T$_{CRD8}^1$ cells. VV-OVA was also nonimmunogenic in Kbm1 mice. As a positive control, mice were infected with two rVVs, one expressing wild-type K$^b$ and mouse b$_2$ m (VV-K$^b$b$_2$ m), the other expressing S-OVA$_{257-264}$ and mouse b$_2$ m (VV-S-OVA$_{257-264}$ b$_2$ m). Under these conditions, where cells doubly infected could express OVA$_{257-264}$ in complex with wild-type H-2K$^b$, a strong proliferative response was observed (Fig. 5). Each of the rVVs alone was nonimmunogenic under these conditions. These findings provide strong evidence that first priming in
the OT-1 transfer system is based on presentation of Ag by TAP1−/− APCs and second that virus-infected APCs present endogenous ER-targeted peptides to T CD8+.

**Cross-priming of rVV-encoded Ags in vivo**

The discrepancy in the presentation of OVA by TAP1−/− cells in vivo and in vitro is consistent with the occurrence of a cross-priming mechanism in which an OVA257–264-containing protein produced by rVV-infected cells is presented by TAP1−/− cells to OT-1 T CD8+ . To explore this possibility, we immunized TAP1−/− mice with splenocytes from B6 or K bm1 mice infected with rVVs encoding OVA, the cytosolic minigene product, or a control rVV.

Immunization of TAP−/− mice with either B6 or K bm1 splenocytes expressing virus-encoded OVA or OVA257–264, but not an irrelevant gene product, activated transferred OT-1 T CD8+ (Fig. 6). Splenocytes are poorly infected by VV, and it is unlikely that they are capable of producing sufficient virus for priming, particularly because relatively large amounts of VV-OVA (10⁵ PFU) are needed to activate OT-1 cells in vivo (data not shown). It is possible, however, that virus is transferred by contact of infected cells with host APCs. To demonstrate that priming was not due to virus transfer, we took advantage of the fact that minigenes offer an exceedingly small target for psoralen-enhanced inactivation by UV light (35). By titrating the time of UV irradiation, we could completely inactivate the ability of VV to replicate while maintaining its capacity to synthesize cytosolic minigenes (although at reduced levels relative to untreated virus). Immunization of mice with cells expressing either K b or K bm1 and infected with UV-irradiated VV-OVA257–264 resulted in activation of OT-1 cells, demonstrating that true cross-priming occurs with the cells expressing a cytosolic minigenie. The activation of OT-1 cells was clearly less than when using noninactivated virus. Whether due to decreased expression of the minimal determinant, decreased VV-induced alterations in infected cells, or spread of VV from cells infected with noninactivated virus is uncertain.

**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 T CD8+. 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 T CD8+ are activated in TAP−/− mice by immunization with VV-OVA-infected K bm1 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 T CD8+ 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 (36). 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 T CD8+.
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 OVA257–264 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 OVA257–264-specific TCD8+ was completely TAP-dependent, inasmuch as the virus did not activate naïve TCD8+ in irradiated B6 mice receiving bone marrow from TAP1−/− mice (42). In the same study, the presentation of VV-S-OVA257–264 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 TCD8+. Indeed, we demonstrate that VV-S-OVA257–264 provides a stronger in vivo stimulus than OVA, and no doubt had we transfected less OT-1 cells we could have set the system to observe a positive response to VV-S-OVA257–264 and negative response to VV-OVA. Similarly, our failure to demonstrate responses to various rNVs in TAP1−/− 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-OVA257–264) as well as the endoproteolytic liberation of NP366–374 from a secreted fragment of NP to adoptively transferred TCD8+ cells expressing the cytosolic OVA257–264 (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 TCD8+ 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 TCD8+. 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 TCD8+ 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 OVA257–264 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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