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Major histocompatibility complex class I presentation of ovalbumin peptide 257–264 from exogenous sources: protein context influences the degree of TAP-independent presentation

Peritoneal macrophages from C57BL/6 mice process antigens from bacteria or coated on polystyrene beads for presentation by major histocompatibility complex (MHC) class I molecules. To investigate this antigen processing pathway, peritoneal macrophages from homozygous *TAP1*$^{-/-}$ mice, which lack the transporter associated with antigen processing (TAP) and are defective in presenting endogenous antigens on MHC class I, were used. *TAP1*$^{-/-}$ or C57BL/6 macrophages were co-incubated with either bacteria or polystyrene beads containing the 257–264 epitope from ovalbumin [OVA(257–264)], which binds the mouse class I molecule K^b. The source of the OVA(257–264) epitope was either the Crl-OVA(257–264) (Crl-OVA) fusion protein, the maltose binding protein (MBP)-Crl-OVA fusion protein, native OVA or bacterial recombinant OVA (rOVA); Crl-OVA, MBP-Crl-OVA and rOVA were each expressed in bacteria, and Crl-OVA and MBP-Crl-OVA purified from bacterial lysates and native egg OVA were coated onto polystyrene beads. The data reveal that peritoneal macrophages from C57BL/6 and *TAP1*$^{-/-}$ mice can process bacteria expressing Crl-OVA, MBP-Crl-OVA and rOVA as well as beads coated with native OVA, purified Crl-OVA, and purified MBP-Crl-OVA and present OVA(257–264) for recognition by OVA(257–264)/K^b-specific T hybridoma cells, albeit with different relative processing efficiencies. The processing efficiency of *TAP1*$^{-/-}$ macrophages co-incubated with bacteria or beads containing Crl-OVA or MBP-Crl-OVA was reduced approximately three to five times compared to C57BL/6 macrophages, but OVA(257–264) was presented 100 times less efficiently when the source of OVA(257–264) was full-length OVA. Chloroquine inhibition studies showed a differential requirement for acidic compartments in C57BL/6 versus *TAP1*$^{-/-}$ macrophages, which also depended upon the source of the OVA (257–264) epitope (Crl-OVA versus full-length OVA). These data suggest that *TAP1*$^{-/-}$ and C57BL/6 macrophages may process Crl-OVA and full-length OVA in different cellular compartments and that the protein context of the OVA(257–264) epitope influences the extent of TAP-independent processing for MHC class I presentation.

1 Introduction

Antigen processing and presentation, a process by which proteins are degraded into peptides and presented on the surface of eukaryotic cells for recognition by T cells, is a critical first step in development of an immune response. Two pathways of antigen processing and presentation exist: in general, peptides originating from endogenous proteins are presented by MHC class I molecules, while peptides generated from exogenous sources are presented by MHC class II molecules (reviewed in [1]). However, reports demonstrating that peptides derived from endogenous proteins can be presented by MHC class II molecules [2–4] and peptides derived from exogenous sources can be presented by MHC class I molecules [5–19] suggest that these two pathways may not be completely isolated from each other. Antigen presentation pathway crossover may provide alternative mechanisms for presenting peptides.

The majority of MHC class I-associated peptides are either peptides of self or viral origin derived from proteins synthesized in the cytosol (reviewed in [20, 21]). Cytosolic proteins are degraded primarily by the proteosome, transported into the lumen of the endoplasmic reticulum by the transporter associated with antigen processing (TAP) and bind newly synthesized MHC class I heavy chain/β2-microglobulin (β_2m) heterodimers; the peptide/β_2m/class I heavy chain complexes are then transported to the cell surface for T cell recognition.

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Abbreviations: TAP: Transporter associated with antigen processing **Crl:** Bacterial cytoplasmic regulatory protein **OVA(257–264):** Amino acids 257–264 of chicken egg ovalbumin **Crl-OVA:** Crl-OVA (residues 257–264) fusion protein **MBP-Crl-OVA:** Maltose binding protein-Crl-OVA fusion protein **β_2m:** β2-microglobulin **rOVA:** Recombinant full-length OVA expressed in bacteria

Key words: Transporter associated with antigen processing (TAP) / Antigen processing and presentation / Major histocompatibility complex class I molecule / Ovalbumin

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The importance of the TAP transporter in presenting endogenous antigens on MHC class I molecules is underscored by the phenotype of cell lines with mutations in the *TAP1, TAP2*, or both loci, such as the mouse T lymphoma cell line RMA-S and the human B lymphoblastoid cell line 721.174 (reviewed in [20]). These cells express extremely low levels of surface class I molecules and cannot present endogenous proteins on MHC class I molecules. Splenocytes from mice with the *TAP1* locus specifically interrupted are deficient in presenting endogenous antigens for recognition by $CD8^+$ CTL and do not express significant levels of surface class I (although class I expression can be rescued by incubation at 26 °C or addition of exogenous peptide). Such mice almost completely lack $CD4^-CD8^+$ T cells, while maintaining normal numbers of $CD4^+CD8^-$ T cells [22]. Thus, in the absence of the TAP transporter, peptide availability in the endoplasmic reticulum (ER) is compromised, leading to a significant reduction of properly folded MHC class I molecules at the cell surface and defective presentation of endogenous antigens by MHC class I molecules.

Although the majority of MHC class I-associated peptides are derived from proteins synthesized by the antigen-presenting cell itself, several studies demonstrated that exogenous particulate antigens can be presented by MHC class I molecules (reviewed in [23]). We have previously shown that mouse macrophages can process bacteria which are not known to escape the phagosome and present bacterial peptides on MHC class I molecules [12, 13]. These studies were performed using a model epitope, the MHC class I-binding 257–264 epitope from ovalbumin [OVA(257–264)] expressed as a fusion protein [Crl-OVA(257–264), or simply Crl-OVA] in the cytosol of bacteria. To investigate further the pathway involved in processing of exogenous antigens containing OVA(257–264) for presentation by the mouse MHC class I molecule K^b and to determine the role of post-Golgi MHC class I molecules, the current study examines the ability of peritoneal macrophages from $TAP1^{-/-}$ mice to process and present antigens expressed by bacteria or coupled to polystyrene beads. Our data demonstrate that the OVA(257–264) epitope is presented with a differential dependence on the TAP transporter depending on the protein context of the OVA epitope: OVA(257–264) contained within the MBP-Crl-OVA or Crl-OVA bacterial fusion proteins is presented with little dependence on the TAP transporter, while OVA(257–264) contained within full-length ovalbumin is largely dependent on the TAP transporter, regardless of whether recombinant OVA is expressed in bacteria or the native protein is coupled to polystyrene beads.

2 Materials and methods

2.1 Antigens: bacterial expression of Crl-OVA, MBP-Crl-OVA and full-length OVA

Table 1 summarizes the bacterial strains used in these studies. *Escherichia coli* HB101/pJLP-2H [12] or *Salmonella typhimurium* χ3000/pJLP-2H with rough LPS [24] were used in experiments with bacteria expressing the Crl-OVA fusion protein. The plasmid pJLP-2H contains the DNA encoding residues 257–277 of chicken egg OVA, including residues 257–264 bound by the mouse H-$2K^b$ molecule [25], fused near the C terminus of the cytoplasmic bacterial regulatory protein Crl, creating the fusion protein Crl-OVA [12]. The Crl protein is a bacterial regulatory protein and is expressed in the bacterial cytoplasm [26]. The OVA(257–264) epitope is internal in the fusion protein, so presentation requires at least one proteolytic cleavage on each side of the 257–264 epitope. Bacteria expressing Crl-OVA were grown overnight at 37 °C on Luria agar plates supplemented with 50 μg/ml ampicillin. Bacterial suspensions were made in PBS pH 7.4 and quantitated by determining the OD at 600 nm; bacterial suspensions were then serially diluted in RPMI medium containing 200 mM L-glutamine, 100 mM sodium pyruvate and 0.1 mM 2-mercaptoethanol and used in antigen processing assays (Sect. 2.3).

Bacteria expressing full-length rOVA were made by PCR amplification of a 1.2-kb fragment from the chicken OVA cDNA contained in pOV230 [27]. The oligonucleotide primers incorporated an Eco RI site 5′ of the OVA start codon and a Bam HI site 3′ of the stop codon. The resultant PCR product was cloned into the corresponding sites of pUHE21-2 [13]. The resulting plasmid, pUHE-OVA, was digested with Hind III and Xho I, the 1.2-kb fragment was purified after agarose gel electrophoresis, cloned into the Hind III and Sal I sites of pUC18 and transformed into competent *E. coli* HB101. The proper DNA insert of the resulting plasmid, called pOVA, was demonstrated by restriction endonuclease analysis; expression of a 46-kDa protein that specifically reacted with rabbit anti-OVA antiserum was confirmed by Western blot analysis. Expression of rOVA in pOVA is under the control of the *lac* promoter/operator; isopropyl β-D-thiogalactopyranoside (IPTG) induction is not required for expression (data not shown). Bacteria expressing rOVA were grown overnight at 37 °C in Luria broth supplemented with 50 μg/ml ampicillin and bacterial suspensions were prepared as described above.

Bacteria expressing the maltose-binding protein (MBP)-Crl-OVA fusion protein were made using the pMAL protein fusion and purification system (New England Biolabs, Beverly, MA). A 440-bp DNA fragment was amplified by PCR from pJLP-2H [12] using oligonucleotide primers that incorporated an Eco RV site 5′ of the Crl start codon and a Bam HI site 3′ of the stop codon [26]. This strategy resulted in an in-frame fusion of MBP to the N terminus of Crl-OVA after cloning the fragment into the Xmn I and Bam HI sites of pMAL-c2. In pMAL-c2, the signal sequence of *malE* (which encodes MBP) is deleted, so the fusion protein is expressed in the bacterial cytoplasm. For purification of the fusion protein as well as for use of *E. coli* expressing MBP-Crl-OVA in antigen processing experiments, recombinant bacteria (*E. coli* DH5α/pMalE-Crl-OVA) were grown and induced to express the fusion protein according to the manufacturer's recommendation; expression of the appropriate 60-kDa MBP-Crl-OVA fusion protein was confirmed by Western blot analysis using both rabbit anti-MBP sera and mouse anti-OVA sera. For antigen processing assays, bacterial suspensions were prepared as described above.

2.2 Antigens: bead OVA, bead MBP-Crl-OVA and bead Crl-OVA preparations

Bead-OVA was prepared by coupling native OVA (Sigma, St. Louis, MO) to carboxylated polystyrene beads (Polysciences, Warrington, PA) using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride as recommended by the manufacturer; the beads were extensively washed following the coupling reactions. Coupling reactions contained 400 μg OVA and used either 1-μm or 3-μm diameter beads. Similar to the 257–264 epitope in the Crl-OVA fusion protein, the 257–264 epitope within full-length OVA requires at least one processing event on each side of the epitope to be liberated.

Bead MBP-Crl-OVA and Crl-OVA preparations were made using purified MBP-Crl-OVA or Crl-OVA, respectively, prepared using the pMAL-c2 system as recommended by the manufacturer (New England Biolabs). MBP-Crl-OVA was purified by affinity chromatography on amylose resin; an aliquot was used as purified MBP-Crl-OVA. To purify Crl-OVA, MBP was released from MBP-Crl-OVA by cleavage with clotting factor Xa. SDS-PAGE was used to analyze the purity of MBP-Crl-OVA and Crl-OVA. These purified proteins were coupled to 3-μm carboxylated polystyrene beads (Polysciences) as described above.

2.3 Antigen processing assay

Homozygous *TAP1*$^{-/-}$ breeding pairs (bred onto a C57BL/6 background [22]) were kindly provided Luc Van Kaer (Vanderbilt Medical School, Nashville, TN). C57BL/6 mice (H-2^b) were purchased from The Jackson Laboratory (Bar Harbor, ME). *Listeria monocytogenes*-elicited peritoneal macrophages were collected from C57BL/6 or homozygous *TAP1*$^{-/-}$ mice and were used in antigen processing assays as described [12, 13]. Briefly, macrophages (2×10^5/well) were allowed to adhere to 96-well tissue culture plates for 2 h at 37 °C. Nonadherent cells were then washed away. Bacteria expressing MBP-Crl-OVA, Crl-OVA or rOVA, or polystyrene beads coated with purified MBP-Crl-OVA, Crl-OVA or native OVA, were then added in antibiotic-free medium. After gentle centrifugation (1000 × g for 5 min), the cultures were incubated at 37 °C for 2 h. Afer washing, the cells were fixed in 1% paraformaldehyde, extensively washed and 10^5 CD8OVA T hybridoma cells [12], which secrete IL-2 upon specific recognition of the OVA(257–264)K^b complex, were added for 24 h. IL-2 production by the CD8OVA T hybridoma cells was quantitated using IL-2-dependent CTLL cells. Where appropriate, cytochalasin D or chloroquine (Sigma) were added at 10 μg/ml and 100 μM, respectively, from 30 min to 1 h prior to addition of bacteria or beads, and were present at 5 μg/ml and 50 μM, respectively, during the antigen processing incubation.

2.4 Flow cytotometry analysis

Single-cell suspensions of splenocytes from individual *TAP1*$^{-/-}$ or C57BL/6 mice were prepared by standard procedures [24]; cells were stained with FITC-labeled hamster anti-mouse CD3ε (Pharmingen, San Diego, CA) and PE-labeled rat anti-mouse CD8a (Caltag, South San Francisco, CA) on ice for 30 min. At least 10000 viable cells were analyzed per experiment using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Routine analysis demonstrated a near total absence of CD8$^+$ T cells from spleens of individual *TAP1*$^{-/-}$ mice (data not shown), as predicted [22], conforming that the phenotype of the *TAP1*$^{-/-}$ mice was preserved in the animals bred at our facility.

2.5 Quantitation of OVA in bacterial and bead preparations

The amount of native OVA, Crl-OVA, or MBP-Crl-OVA coupled to the polystyrene beads was determined by quantitating the amount of protein recovered in the supernatant after the coupling reaction as recommended by the manufacturer. The amount of the OVA epitope present in *E. coli* rOVA, *E. coli* Crl-OVA, or *E. coli* MBP-Crl-OVA was quantitated by densitometer scanning of SDS-polyacrylamide gels after electrophoresis of $2.5 \times 10^6 - 5 \times 10^7$ boiled whole bacteria per lane. Measurement of rOVA, Crl-OVA, or MBP-Crl-OVA expression in different bacterial preparations showed that expression varied only slightly, and was consistently within the narrow range of 20–75, 30–100, or 250–830 ng/10^6 bacteria, respectively. Table 2 shows the quantity of native OVA, Crl-OVA or MBP-Crl-OVA coupled to 3-μm polystyrene beads and rOVA, Crl-OVA or MBP-Crl-OVA expressed in bacteria.

2.6 Quantitation of bacteria or beads associated with peritoneal macrophages

Peritoneal macrophages (10^6) from either C57BL/6 or *TAP1*$^{-/-}$ mice were allowed to adhere to wells of 24-well tissue culture plates at 37 °C. Bacteria (titrated in the range of $3 \times 10^6 - 3 \times 10^7$/well) fluorescently labeled with 5(6)-carboxyfluorescein-N-hydroxysuccimide ester (Boehringer-Mannheim, Indianapolis IN) [28], or polystyrene beads (2.5×10^6/well), were incubated with adherent macrophages for 30 min prior to washing and quantitating the number of bacteria or beads associated with the macrophages by fluorescence microscopy.

3 Results

3.1 The degree of TAP-independent processing depends upon the source of the OVA(257–264) epitope

Peritoneal macrophages from C57BL/6 mice phagocytose and process bacteria expressing the Crl-OVA fusion protein and present the OVA(257–264) epitope on mouse K^b molecules [12, 13]. To investigate the antigen processing pathway used for this presentation, the ability of peritoneal macrophages from *TAP1*$^{-/-}$ mice to process and present the OVA(257–264) epitope from *E. coli* expressing different proteins containing this OVA epitope was assessed (Fig. 1). The data show that macrophages from both C57BL/6 and *TAP1*$^{-/-}$ mice process bacteria expressing Crl-OVA, MBP-Crl-OVA or rOVA and present OVA(257–264) on K^b. The efficiency with which *TAP1*$^{-/-}$ macrophages processed and presented the OVA epitope

Table 1. Bacterial strains used in this study

Bacterial strain	Description	Reference
Escherichia coli HB101/pJLP-2H	*E. coli* expressing the Crl-OVA fusion protein	[12]
Salmonella typhimurium χ3000/pJLP-2H	*S. typhimurium* with rough LPS expressing the Crl-OVA fusion protein	[24]
E. coli HB101/pOVA	*E. coli* expressing recombinant OVA	This study
E. coli DH5α/ pMalECrl-OVA	*E. coli* expressing the MBP-Crl-OVA fusion protein	This study

depended upon the source of OVA(257–264). Whereas macrophages from *TAP1*$^{-/-}$ mice processed *E. coli* expressing Crl-OVA or MBP-Crl-OVA (Figs. 1A and B) or *Salmonella typhimurium* expressing Crl-OVA (Fig. 2) and presented OVA(257–264) three to five times less efficiently compared to C57BL/6 macrophages, they processed and presented *E. coli*-expressed rOVA approximately 100 times less efficiently (Fig. 1C). Maximal processing for presentation of OVA(257–264) by both C57BL/6 and *TAP1*$^{-/-}$ macrophages was observed with approximately 60–100 pmoles of the OVA epitope when present in the Crl-OVA fusion protein, while C57BL/6 macrophages reached maximal presentation with 1–5 pmoles OVA(257–264) when present in rOVA (Fig. 1C). In contrast, *TAP1*$^{-/-}$ macrophages processed *E. coli* expressing rOVA and presented OVA(257–264) with relatively poor efficiency (Fig. 1C). Thus, although both C56BL/6 and *TAP1*$^{-/-}$ macrophages clearly can process bacteria expressing a source of OVA(257–264), the protein harboring this

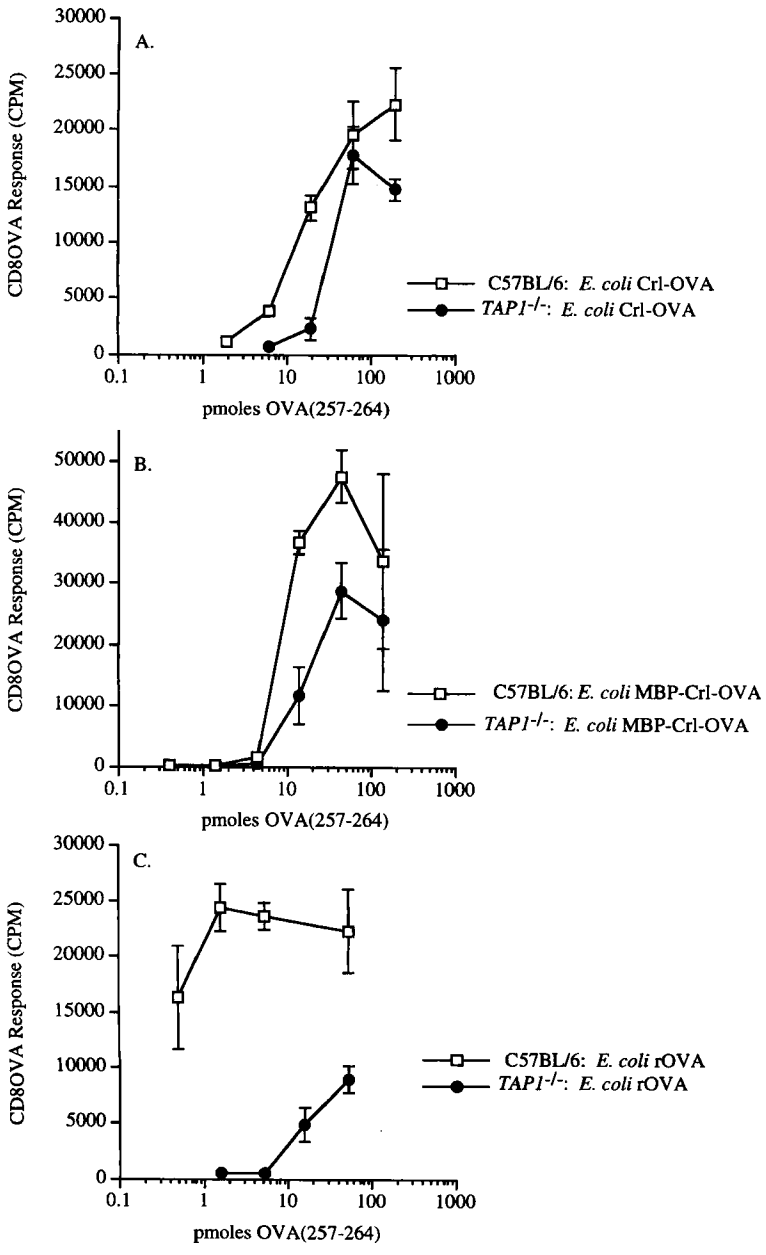

Figure 1. Presentation of OVA(257–264) from bacteria expressing Crl-OVA and MBP-Crl-OVA, but not from bacteria expressing rOVA, is largely TAP independent. The response of CD8OVA T hybridoma cells after coincubation of peritoneal macrophages from either *TAP1*$^{-/-}$ or C57BL/6 mice with *E. coli* expressing the Crl-OVA fusion protein (A), the MBP-Crl-OVA fusion protein (B), or rOVA (C) is shown. The response using peritoneal macrophages from *TAP1*$^{-/-}$ mice is indicated with filled circles while that using macrophages from C57BL/6 mice is indicated with open squares. The antigen processing assays were incubated for 2 h prior to stopping by fixing the macrophages. Data points represent the mean of triplicate samples ± 1 SD and are representative of six independent experiments.

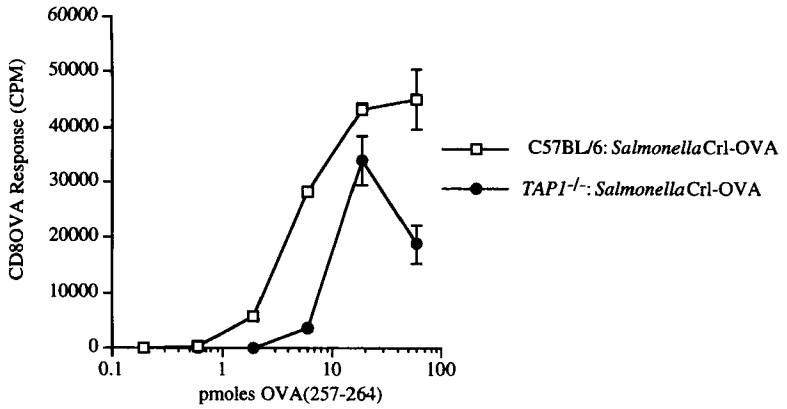

Figure 2. Peritoneal macrophages from C57BL/6 and *TAP1*$^{-/-}$ mice can process *Salmonella typhimurium* χ3000 expressing the Crl-OVA fusion protein and present OVA(257–264) with similar efficiencies. The response of CD8OVA T hybridoma cells after co-incubation of peritoneal macrophages from either *TAP1*$^{-/-}$ or C57BL/6 mice with *S. typhimurium* χ3000/pJLP-2H with rough LPS [24] is shown. The antigen processing assays were incubated for 2 h prior to stopping by fixing the macrophages. The response using peritoneal macrophages from *TAP1*$^{-/-}$ mice is indicated with filled circles and that using macrophages from C57BL/6 mice is indicated with open squares. Data points represent the mean of triplicate samples ± 1 SD and are representative of three independent experiments.

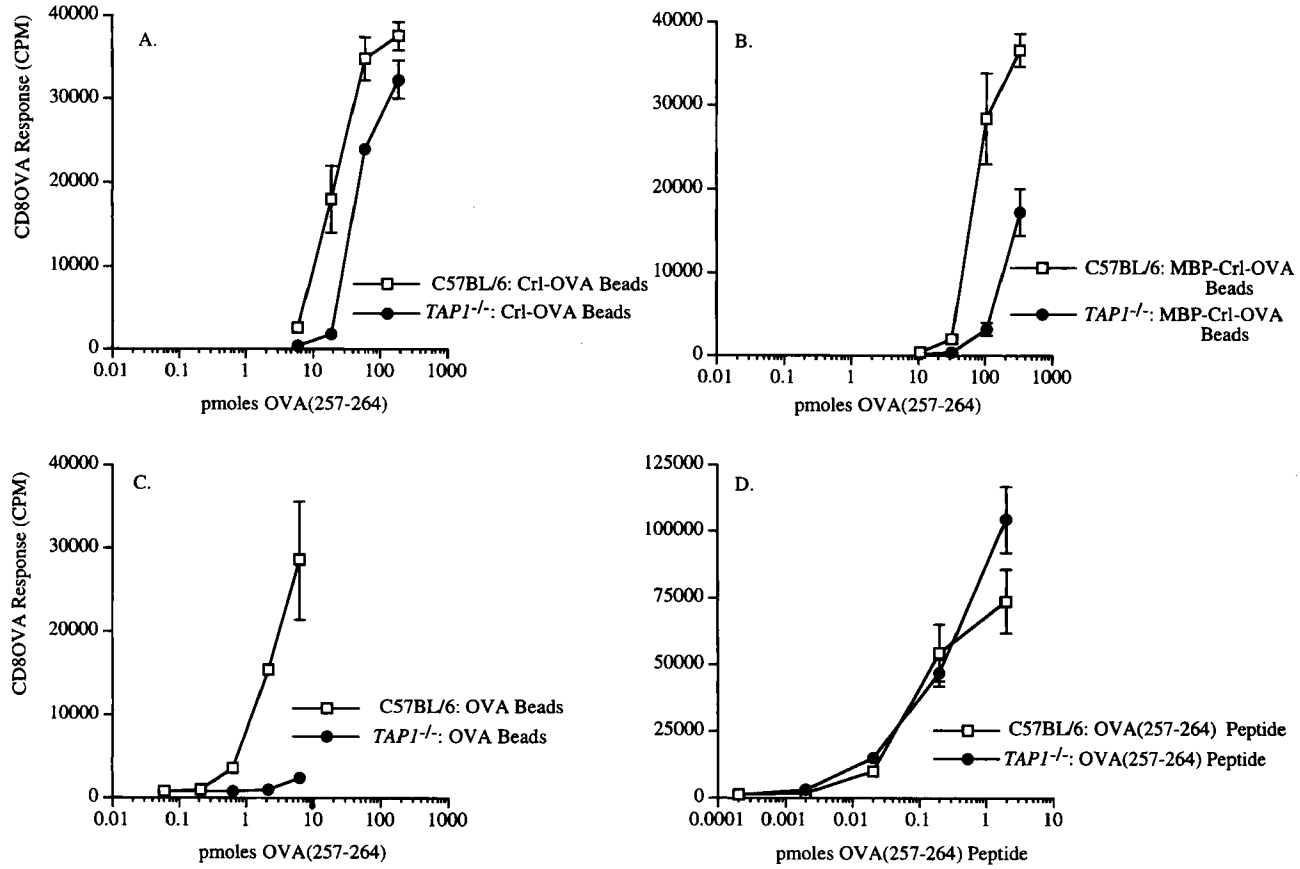

Figure 3. Presentation of OVA(257–264) from polystyrene beads coated with Crl-OVA or MBP-Crl-OVA, but not from beads coated with native OVA, is largely TAP-independent. The response of CD8OVA T hybridoma cells after co-incubation of peritoneal macrophages from either *TAP1*$^{-/-}$ or C57BL/6 mice with 3-μm polystyrene beads coated with purified Crl-OVA (A), MBP-Crl-OVA (B), or native OVA (C) is shown. Identical results for native OVA were observed using 1-μm polystyrene beads (data not shown). The response using peritoneal macrophages from *TAP1*$^{-/-}$ mice is indicated with filled circles and that using macrophages from C57BL/6 mice is indicated with open squares. Addition of exogenous peptide to C57BL/6 or *TAP1*$^{-/-}$ macrophages resulted in similar levels of peptide presentation (D). The antigen processing assays were incubated for 2 h prior to stopping by fixing the macrophages. Data points represent the mean of triplicate samples ± 1 SD and are representative of five independent experiments.

Table 2. Quantitation of OVA(257–264) epitope expressed by recombinant bacteria or coupled to polystyrene beads

Source of OVA(257–264)	Amount of antigen[a)]	OVA(257–264) epitope in wells containing 10^6 bacteria or beads
Escherichia coli Crl-OVA	30–100 ng/10^6 bacteria	2–6 pmoles
Salmonella typhimurium Crl-OVA	30–100 ng/10^6 bacteria	2–6 pmoles
E. coli rOVA	20–75 ng/10^6 bacteria	0.4–1.6 pmoles
E. coli MBP-Crl-OVA	250–830 ng/10^6 bacteria	4.2–14 pmoles
Crl-OVA Beads (3-μm)	330 ng/10^6 beads	19 pmoles
OVA Beads (3-μm)	60 ng/10^6 beads	1.3 pmoles
MBP-Crl-OVA Beads (3-μm)	2000 ng/10^6 beads	33 pmoles

a) See Sect. 2.5 for details of quantitation.

epitope influences the degree of TAP-independent presentation. Cytochalasin D, an inhibitor of actin polymerization and therefore phagocytosis, completely inhibited the presentation of the OVA epitope, regardless of which protein was expressed in the bacteria (Crl-OVA, MBP-Crl-OVA or rOVA; data not shown), demonstrating that phagocytosis was required for presentation of OVA(257–264) from the bacteria by both *TAP1*$^{-/-}$ and C57BL/6 macrophages.

It has been reported that presentation of native OVA coupled to beads is TAP-dependent and occurs by a mechanism requiring the phagocytosed OVA to leak from the phagosome into the cytosol, thereby gaining access to the classical TAP-dependent MHC class I presentation pathway [14, 15, 29]. Fig. 1C shows that *TAP1*$^{-/-}$ macrophages process bacteria expressing rOVA and present OVA(257–264) on K^b with significantly reduced efficiency (approximately 100 times) compared to macrophages from C57BL/6 mice, while they process bacteria expressing Crl-OVA or MBP-Crl-OVA and present OVA(257–264) on K^b only slightly less efficiently than C57BL/6 macrophages (Figs. 1A, B and 2). Since the only difference between the two proteins is the context of the OVA(257–264) epitope (Crl-OVA versus rOVA), and since previous studies showed presentation of native OVA coupled to beads was TAP-dependent [29], we investigated the differential TAP-dependent presentation of the OVA(257–264) epitope from Crl-OVA, MBP-Crl-OVA or native OVA coupled to beads.

We coated polystyrene beads with either purified Crl-OVA, MBP-Crl-OVA or native OVA and used these as the source of OVA(257–264) in antigen-processing assays with macrophages from C57BL/6 or *TAP1*$^{-/-}$ mice. Fig. 3 demonstrates that the OVA(257–264) epitope is processed with only slightly reduced efficiency (approximately three to five times lower) by *TAP1*$^{-/-}$ macrophages that phagocytosed polystyrene beads coated with purified Crl-OVA or MBP-Crl-OVA, while presentation of the same epitope by *TAP1*$^{-/-}$ macrophages from beads coated with native OVA is markedly reduced (10–100 times). Identical results were obtained with either 1-μm (data not shown) or 3-μm polystyrene beads. Presentation of the OVA epitope from polystyrene beads coated with any of the three proteins by *TAP1*$^{-/-}$ and C57BL/6 macrophages was completely inhibited by cytochalasin D (data not shown), demonstrating that phagocytosis of beads was required for epitope presentation. This significant level of TAP-dependent presentation of OVA(257–264) on K^b processed from polystyrene beads coated with native OVA was repeatedly observed in our experiments and agrees with previously published data [29].

The data in Figs. 1–3 are presented as pmoles of OVA epitope present in the bacteria or beads added to the assays and underscore the observation that epitope abundance did not account for the relative difference in presentation efficiency after processing of Crl-OVA, MBP-Crl-OVA, rOVA, or native OVA, from either beads or bacteria (Table 2). Quantitation of antigen conjugated to polystyrene beads and that expressed in bacteria showed that the antigen processing efficiency by wild-type macrophages was greatest with native OVA [maximal response required 1–6 pmoles OVA(257–264); Figs. 1C and 3C]. In contrast, macrophages from *TAP1*$^{-/-}$ mice required between 10–100 pmol epitope from native OVA, and this level resulted in significantly lower processing and presentation compared to C57BL/6 macrophages. These data suggest that, while some presentation of OVA(257–264) occurs when *TAP1*$^{-/-}$ macrophages phagocytose a source of OVA, whether native or recombinant, this presentation is largely dependent on the TAP transporter. In contrast, C57BL/6 and *TAP1*$^{-/-}$ macrophages process bacteria or beads harboring OVA(257–264) in Crl-OVA or MBP-Crl-OVA with similar efficiencies (Figs. 1A, B; 2; 3A, B), demonstrating that processing of Crl-OVA and presentation of OVA(257–264) is largely independent of the TAP transporter. In addition, the observed difference in antigen processing efficiency was not due to differential phagocytosis of beads versus bacteria, since similar numbers of beads or bacteria were associated with macrophages from either C57BL/6 or *TAP1*$^{-/-}$ mice (Table 3). These data suggest that two different processing pathways for presentation of exogenous antigens on MHC class I exist: one that is largely TAP-dependent and another that is largely

Table 3. Quantitation of bacteria or polystyrene beads associated with peritoneal macrophages from *TAP1*$^{-/-}$ or C57BL/6 mice[a)]

	Source of macrophages	
	C57BL/6	*TAP1*$^{-/-}$
Escherichia coli HB101	9.1 ± 4.0	11.4 ± 4.1
3-μm Beads	11.9 ± 7.1	13.3 ± 7.6

a) Polystyrene beads or fluorescently-labeled bacteria were incubated with macrophages as indicated in Sect. 2.6. Values are expressed as the mean number of beads or bacteria associated per individual macrophage ± 1 SD. A minimum of 50 macrophages were counted in each experiment.

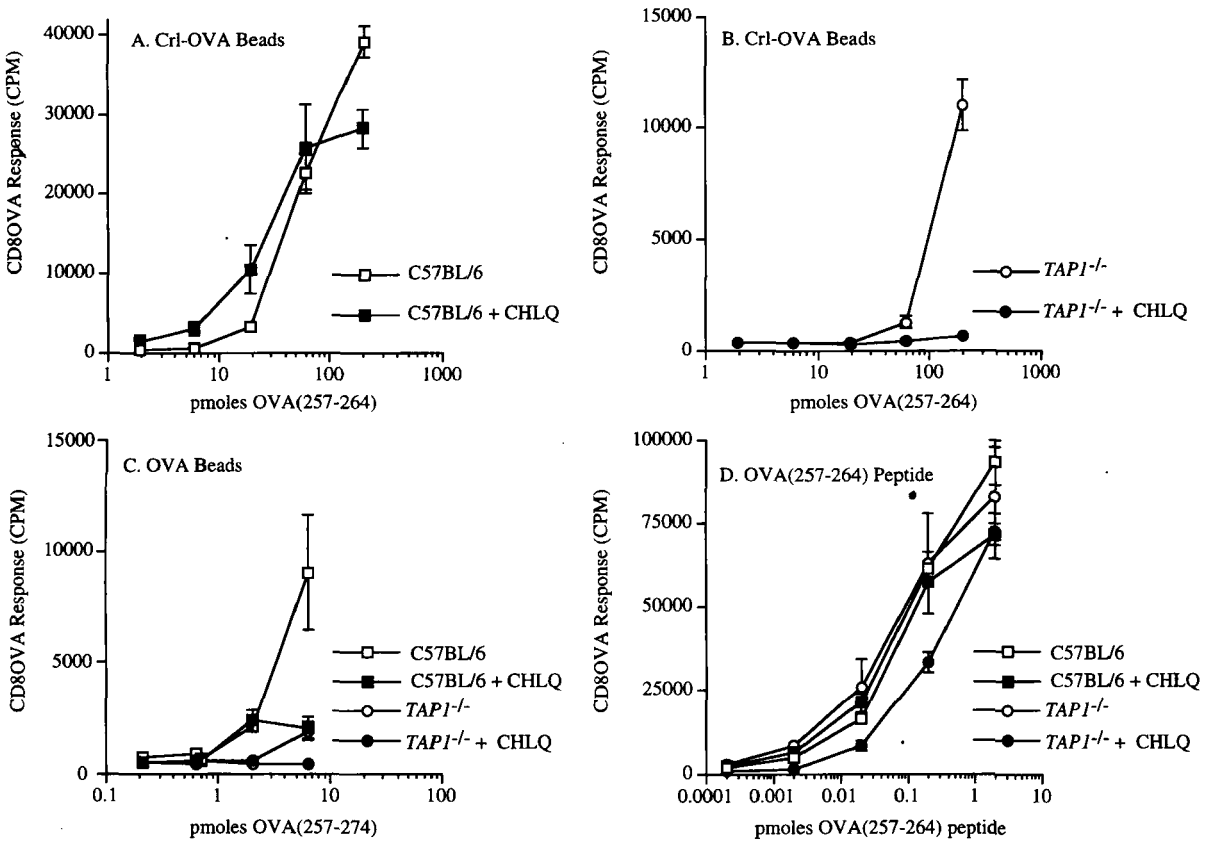

Figure 4. The role of acidic compartments in presenting OVA(257–264) from exogenous sources. Peritoneal macrophages from C57BL/6 or *TAP1*$^{-/-}$ mice were either treated with 100 μM chloroquine (CHLQ) or were left untreated as indicated. CHLQ was added 1 h before addition of the beads or peptide and was present during the entire 2-h experiment at 50 μM. The response using peritoneal macrophages from *TAP1*$^{-/-}$ mice is indicated with circles and that using macrophages from C57BL/6 mice is indicated with squares; CHLQ addition is indicated by filled symbols. The effect of chloroquine on presentation of 3-μm Crl-OVA beads (A and B), on 3-μm native OVA beads (C) and on exogenous OVA(257–264) peptide (D) is shown. The antigen processing assays were incubated for 2 h prior to stopping by fixing the macrophages. Data points represent the mean of triplicate samples ± 1 SD and are representative of three independent experiments.

independent of the TAP transporter. Furthermore, the source of the OVA epitope determines which pathway predominates.

The observed processing and presentation was epitope-specific, as *E. coli* expressing the irrelevant epitope HEL(52–61) [30] evoked no response by the CD8OVA T hybridoma cells (data not shown). Addition of exogenous peptide restored full presentation capability of peritoneal macrophages from *TAP1*$^{-/-}$ mice compared with C57BL/6, as expected [22] (data not shown). In summary, peritoneal macrophages from *TAP1*$^{-/-}$ mice, which do not express stable, peptide-loaded MHC class I molecules and cannot present cytosolic antigen [22], efficiently process exogenous particulate antigens and present OVA(257–264) from Crl-OVA or MBP-Crl-OVA, but inefficiently present OVA(257–264) from native or recombinant OVA. This suggests that the protein context of OVA(257–264) influences the degree of TAP-independent presentation.

3.2 The role of endosomal compartments in processing of exogenous sources of OVA(257–264) for MHC class I presentation

The role of acidic compartments in the processing of OVA(257–264) from native OVA or Crl-OVA coupled to polystyrene beads was assessed in experiments using the weak basic amine chloroquine (Fig. 4). Addition of 100 μM chloroquine to antigen processing experiments with C57BL/6 macrophages co-incubated with Crl-OVA beads had little effect on the presentation of OVA(257–264) to CD8OVA T hybridoma cells (Fig. 4A). In contrast, chloroquine addition resulted in reduced antigen processing efficiency when *TAP1*$^{-/-}$ macrophages phagocytosed Crl-OVA beads (Fig. 4B). Furthermore, the presence of chloroquine resulted in reduced presentation of OVA(257–264) when C57BL/6 or *TAP1*$^{-/-}$ macrophages phagocytosed OVA beads (Fig. 4C). However, the effect of chloroquine on presentation of native OVA coupled to beads must be interpreted with the caveat that we were unable to add higher concentrations of native OVA coupled to beads due to limitations in the amount of OVA that we could couple to the beads; the data shown in Fig. 4C results from the use of the maximal amount of OVA coupled to beads and the maximal number of beads added to the wells. Addition of chloroquine to macro-

phages exposed to OVA(257–264) peptide had no effect (Fig. 4D). In summary, the processing of Crl-OVA for OVA(257–264) presentation on K^b by *TAP1*$^{-/-}$ macrophages was significantly reduced in the presence of chloroquine, while chloroquine had little in influence on the presentation of OVA(257–264) from Crl-OVA by C57BL/6 macrophages. Chloroquine also effected the presentation of OVA(257–264) from native OVA beads by both *TAP1*$^{-/-}$ and C57BL/6 macrophages. Together, these data suggest that the antigen-processing pathway utilized when *TAP1*$^{-/-}$ macrophages process Crl-OVA or native OVA for OVA(257–264) presentation on K^b requires passage through acidic compartments. In contrast, acidic compartment(s) do not seem to be involved when C57BL/6 macrophages process Crl-OVA beads, while presentation of the OVA epitope from native OVA may involve acidic compartments in these cells.

4 Discussion

We have previously shown that C57BL/6 macrophages can process bacteria expressing the Crl-OVA fusion protein for presentation of OVA(257–264) by K^b utilizing a pathway that did not require newly synthesized MHC class I molecules: the process was shown to be brefeldin A-resistant [12]. To asses the role of surface or post-Golgi MHC class I molecules in this antigen processing pathway, we utilized macrophages from *TAP1*$^{-/-}$ mice as antigen-presenting cells in the present study. Features of cells from *TAP1*$^{-/-}$ mice such as defective intracellular transport of MHC class I molecules and significantly reduced surface MHC class I expression [22] make them an ideal model system to characterize the pathway by which exogenous particulate antigens are presented by MHC class I molecules.

Our results demonstrate that macrophages from *TAP1*$^{-/-}$ mice can process bacteria or polystyrene beads containing the OVA(257–264) epitope for presentation on K^b (Figs. 1–3). We also demonstrate that the protein context of the OVA(257–264) epitope influences the degree of TAP-independent presentation. Importantly, the differential degree of TAP-independent presentation of OVA(257–264) from Crl-OVA or native OVA is not due to the physical nature of the antigen source, differential phagocytosis (Table 3), or differential epitope abundance (Table 2; see also Figs. 1–3). We also provide clear evidence that presentation of OVA(257–264) contained within the fusion proteins Crl-OVA or MBP-Crl-OVA occurs only slightly less efficiently in the absence of the TAP transporter (Figs. 1A, B; 2; 3A, B). Notably, this same degree of TAP-independent presentation occurs whether Crl-OVA is expressed within different bacteria (*E. coli* or *S. typhimurium*), within different-sized fusion proteins (MBP-Crl-OVA or Crl-OVA) or coupled to 1-μm or 3-μm polystyrene beads. Taken together, these data suggest that the protein context of the OVA(257–264) epitope determines whether its presentation occurs largely by a TAP-dependent or TAP-independent pathway. The largely TAP-dependent presentation of OVA(257–264) from native OVA presented here is consistent with other reports demonstrating that native OVA accesses the cytosol of the antigen-processing cell and can be presented by MHC class I molecules [14, 15, 29].

One possible explanation for the differential TAP-dependent presentation of OVA(257–264) from native OVA versus Crl-OVA may be processing efficiency influenced by flanking residues, protein conformation, or both. Although flanking residues have been shown to influence antigen processing efficiency of MHC class I peptides derived from endogenous antigens [31, 32], the influence of flanking residues on the processing efficiency of exogenous antigens for MHC class I presentation is unknown. Alternatively, Crl-OVA and native OVA may differ in the efficiency by which they are processed by the proteosome. Although recent data suggests that the proteosome is not required for presentation of OVA(257–264) when C57BL/6 macrophages process either Crl-OVA from bacteria or OVA-coated beads [33], the relative processing efficiency of these two antigens by the proteosome was not directly assessed. The different sources of OVA(257–264) (Crl-OVA versus native OVA) may also be processed in different cellular compartments, may differ in their ability to gain access to different cellular compartments, or enter the same compartments with different efficiencies. For example, native ovalbumin has been shown to traverse membranes due to an internal region of this protein [34]. This feature of OVA may allow it more efficient access to the classical MHC class I presentation pathway compared to Crl-OVA.

Our data show that presentation of OVA(257–264) from Crl-OVA by *TAP1*$^{-/-}$ macrophages involves acidic intracellular compartments (Fig. 4B), while the presentation of OVA(257–264) from the same antigens by C57BL/6 macrophages is less dependent on acidic compartments (Fig. 4A). These data suggest that, while a similar pathway for processing exogenous particulate antigens requiring acidic compartments may be present in C57BL/6 macrophages, it is masked by the previously identified TAP-dependent cytosolic escape pathway [29]; in the absence of the classical MHC class I presentation pathway, as is the case in *TAP1*$^{-/-}$ macrophages, the role of acidic compartments in processing exogenous particulate antigens for MHC class I presentation dominates. Furthermore, the requirement for acidic compartments for different sources of OVA(257–264) (Crl-OVA versus native OVA) differs when C57BL/6 macrophages are the antigen-presenting cell: presentation of OVA(257–264) from Crl-OVA does not require acidic compartments (Fig. 4A), while presentation of OVA(257–264) from native OVA does (Fig. 4C). However, we were unable to couple higher levels of OVA to the beads, which prevented examining the effect of chloroquine using quantities of OVA(257–264) in native OVA as high as we could using Crl-OVA.

Although our data suggest that acidic compartments may be involved in the processing of native for OVA(257–264) presentation on K^b, others have shown that chloroquine does not inhibit the processing of native OVA coupled to beads [29] or soluble OVA added in the presence of beads [14]. The role of endosomal compartments in the processing of exogenous OVA for MHC class I presentation is further complicated by reports that some inhibitors of endosomal function (ammonium chloride and leupeptin [14]) inhibit the presentation of the OVA epitope derived from soluble OVA added in the presence of beads. Thus, although endosomal compartments have been shown to be required for the processing of other exogenous antigens

for TAP-independent, brefeldin A-resistant presentation by MHC class I [18, 35, 36], the role of different cellular compartments in the processing of exogenous sources of OVA(257–264) for MHC class I presentation remains unclear.

Since the mechanism(s) of loading peptides derived from exogenous proteins onto MHC class I molecules is not clear, an intriguing question remains: where and how do peptides from exogenous antigens bind MHC class I molecules? Peptides may be loaded onto the pre-existing surface MHC class I molecules by a peptide regurgitation mechanism [12] and, as *TAP1*$^{-/-}$ macrophages have significantly reduced surface MHC class I expression [22], reduced presentation by *TAP1*$^{-/-}$ macrophages may merely reflect decreased levels of surface MHC class I. In support of this hypothesis, addition of β_2m to *TAP1*$^{-/-}$ macrophages, which stabilizes surface MHC class I molecules, enhanced the presentation of OVA(257–264) from exogenous sources [33]. However, while we and others have demonstrated that peptides from exogenous antigens can be loaded onto MHC class I at the macrophage surface [10, 12, 37], others have not found peptide regurgitation to be involved [11, 14, 38], or found that the physical nature of the antigen itself influences whether regurgitation of peptides occurs [19]. This is further complicated by conflicting reports of regurgitation using the same antigen, OVA beads: whereas one group observed peptide regurgitation from OVA beads [10], others did not [11, 14]. These discrepancies may be due, for example, to differing peptide contaminants in different OVA preparations [14] or to different sensitivities of the experimental systems (*i.e.* the T cell hybridomas used).

It is also possible that peptides derived from exogenous sources are loaded onto MHC class I molecules in endosomal/phagolysosomal compartments. In support of this hypothesis, the invariant chain has been shown to associate with a subset of MHC class I molecules and to direct them into endosomal compartments [39]. An alternative means by which peptides from exogenous antigens could be loaded onto MHC class I molecules could be the small number of MHC class I molecules present in the trans-Golgi network, which intersect with endocytic compartments containing degraded antigens. Thus, peptides from degraded phagocytosed particles could be loaded onto MHC class I molecules in phagosomal, phagolysosomal or endosomal compartments that contain both antigen degradation products and MHC class I molecules. MHC class I molecules could be present in such compartments due to the recycling of stable or unstable MHC class I complexes from the cell surface [40–43], internalization of surface MHC class I complexes during phagocytosis of the antigen, or trafficking of MHC class I to endosomal compartments mediated by the invariant chain [39].

TAP-independent presentation of other exogenous antigens has been described. For example, the recombinant glycoprotein and nucleoprotein from lymphocytic choriomeningitis virus and the nucleoprotein of vesicular stomatitis virus are presented on MHC class I independently of the TAP transporter [38]. Heat-inactivated Sendai virus lacking fusion and hemagglutinin-neuraminidase activities accesses the MHC class I presentation pathway in a TAP-independent fashion [44], and hepatitis B virus small surface antigen particles and cytoplasmic forms of the simian virus 40 large T antigen are processed in endosomal compartments for MHC class I presentation independently of the TAP transporter [18, 35].

In summary, the data presented here confirm the presence of two distinct pathways for the presentation of OVA(257–264) processed from exogenous antigens. One pathway is largely TAP dependent and occurs when OVA(257–264) is processed from native or recombinant OVA. The other pathway is largely TAP independent and occurs when OVA(257–264) is present in Crl-OVA or MBP-Crl-OVA. Both pathways may operate simultaneously and features of the exogenous antigen itself, such as differential processing efficiency determined by local protein context of the epitope or different intracellular trafficking, may influence whether the classical MHC class I presentation pathway or an alternative MHC class I presentation pathway is predominantly used.

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