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CUTTING EDGE

Cutting Edge: Efficient MHC Class I Cross-Presentation during Early Vaccinia Infection Requires the Transfer of Proteasomal Intermediates between Antigen Donor and Presenting Cells[1]

Amparo Serna, Maria C. Ramirez,[2] Anna Soukhanova,[3] and Luis J. Sigal[4]

Priming of CD8$^+$ T cells requires presentation of short peptides bound to MHC class I molecules of professional APCs. Cross-presentation is a mechanism whereby professional APC present on their own MHC class I molecules peptides derived from degradation of Ags synthesized by other Ag "donor cells." The mechanism of cross-presentation is poorly understood, and the nature of the transferred Ag is unknown. In this report, we demonstrate that the bulk of a cross-presented Ag transferred from donor cells recently infected with vaccinia virus are proteasomal products that are susceptible to peptidases within the donor cell cytosol and not full-length proteins or mature epitopes either free or bound to chaperones. The Journal of Immunology,* 2003, 171: 5668–5672.

Most cells of the organism can only present MHC class I-restricted Ags using the direct presentation pathway. In direct presentation, proteins synthesized by the cell are mostly degraded in the cytosol by the proteasome. Some of the resulting oligopeptides may or may not be further trimmed by others peptidases and are transported to the endoplasmic reticulum (ER)[5] via the transporter associated with Ag presentation (TAP). Within the ER, the peptides can be further trimmed, bind to nascent MHC class I molecules, and are finally transported to the cell surface for recognition by CD8$^+$ T cells (1).

Although activated CD8$^+$ T lymphocytes find targets in any cell expressing the appropriate class I-peptide complexes, CD8$^+$ T cell priming requires the Ag being presented by professional APCs (pAPC) (2–4). Like most other cells, pAPC can present peptides directly. However, pAPC can also cross-present Ags synthesized by Ag donor cells, a process that might be important to generate CD8$^+$ T cell responses to viruses that either do not infect pAPC or that cripple their Ag-presenting function (3, 5).

The observation that pAPC can present exogenous proteins with MHC class I and induce CD8$^+$ T cells led to the idea that proteins constitute the transferred material (6, 7). In this "protein" model, pAPC acquire Ag as intact or almost intact proteins from donor cells, degrade them to epitopes, and present them via two alternative routes. In the cytosolic route, the proteins in the vacuoles access the cytosol and are processed as in the direct pathway (8). In the vacuolar route, the proteins are degraded within endocytic compartments and the peptides bind to recycling class I molecules without accessing the cytosol (9). The acquisition, processing, and presentation of exogenous protein by pAPC certainly explains the induction of CD8$^+$ T cells by inactivated viruses, virus-like particles, and possibly other microorganisms such as bacteria and intracellular parasites (10).

In the alternative "heat shock protein" (HSP) model of cross-presentation, the Ags are degraded to mature epitopes within the donor cells, the resulting peptides binding to HSP in the cytosol or the ER. The HSP-peptide complexes are taken up by pAPC through the cell surface receptor CD91, internalized, transferred to the cytosol where the peptide cargo is released from HSP to join the classical class I presentation pathway (11). This model is based in the observation that the inoculation of mice with purified cytosolic HSP70 or 90 and ER-resident gp96 obtained from tumors, induced CD8$^+$ T cells specific for those tumors from which the HSP were purified. Moreover, incubation of pAPC with HSP obtained from Ag-bearing cells results in cross-presentation (11). Both, the protein and HSP models represent inferences from data obtained with isolated antigenic material that do not necessarily model the actual transfer of Ags from donor cells to pAPC for which there is no direct data. In this report, we demonstrate that for chicken OVA, proteasomal intermediates and not full-length proteins or the mature epitope either free or bound to chaperones constitute most of the Ag cross-presented by pAPC when acquired from donor cells recently infected with vaccinia virus.

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[5] Abbreviations used in this paper: ER, endoplasmic reticulum; TAP, transporter associated with Ag presentation; pAPC, professional APC; HSP, heat shock protein; β-gal, β-galactosidase; Mϕ, macrophage; TOP, thimet oligopeptidase; LAP, leucyl aminopeptidase.

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Materials and Methods

All cells were maintained in RPMI 1640 medium supplemented with 10% FCS (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine, penicillin-streptomycin, 0.01 M HEPES buffer, and nonessential amino acids (all from Invitrogen, San Diego, CA) and 5×10^{-5} M 2-ME (Sigma-Aldrich, St. Louis, MO). A9 cells (ATCC no. CCL-1.4; American Type Culture Collection, Manassas, VA) are a derivative of the strain L (H-2^K). A9-T7 cells (12, 13) are A9 cells stably transfected with T7 polymerase (a gift from B. Moss, National Institute of Allergy and Infectious Diseases, Bethesda, MD). MC57G (ATCC no. CRL-2295) are a C57BL/6 fibrosarcoma (H-2^b). B3Z (14) is a CTL hybridoma that produces β-galactosidase (β-gal) upon recognition of the OVA epitope 258–265 in the context of the H-2K^b molecule (a gift from N. Shastri, University of California, Berkeley, CA). Hela S3 (CCL-2.2) and BS-C-1 (CCL-26) were obtained from American Type Culture Collection. All cells were grown at 37°C in an atmosphere of 5% CO_2.

Mice

All experiments requiring animals were performed under protocols approved by the Institutional Animal Use and Care Committee. Mice were bred in Fox Chase Cancer Center's Laboratory Animal Facility. TAP$^{0/0}$ (B6-Tap^{1tp1Arp}) breeders were originally purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 (B6) mice were obtained from the Fox Chase Cancer Center colony.

Viruses

Wild-type WR and T7 recombinant vaccinia virus (a gift from B. Moss) were grown and titered following published procedures (13). Virus titers were adjusted to 10^9 PFU in RPMI 1640 medium containing 2.5% FCS.

Plasmids

Full-length OVA cDNA and (M)50–386 were gifts from L. Shen (University of Massachusetts Medical Center, Worcester, MA). Other constructs were generated by recombinant PCR. All constructs contained a Kozac sequence, were cloned into plasmid Bluescript SKII using the *Bam*HI and *Not*I sites (Invitrogen), and were sequenced at the Fox Chase Cancer Center DNA Sequencing Facility. Plasmids pcDNA-thimet oligopeptidase (TOP) and pcDNA-leucyl aminopeptidase (LAP) were gifts from I. York (University of Massachusetts Medical Center, Worcester, MA). pcDNA3-NS5A was a gift from O. Isken (Fox Chase Cancer Center, Philadelphia, PA).

Ag presentation assays

All Ag presentation experiments were repeated at least three times, each figure corresponding to a representative experiment. Data points in the figures represent the average of duplicate wells.

Direct presentation.

MC57G cells (2×10^5) were plated in 24-well plates, incubated overnight, infected for 1 h with recombinant vaccinia-T7 (10 PFU/cell), and transfected with the indicated constructs using lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. When required, 2 μM lactacystin were present in the medium. Four hours later, the cells were harvested, fixed, washed, and serially diluted (1/2) in 96-well plates (10^5 cells/well in the initial dilution). B3Z responder cells (10^5) were added to each well, and incubated for additional 18–24 h to allow for the activation of the hybridoma and production of β-gal. β-gal activity, was determined with the Galactostar chemiluminescent kit (Tropix; Applied Biosystems, Foster City, CA) as before (15).

Cross-presentation

Ag donor cells. As Ag donor cells we used A9-T7 which are a C3H (H-2^k) mouse fibroblast cell line (L cell subclone A9 cells; ATCC no. CCL-1.4) that stably expresses the phage T7 polymerase (A9-T7). These cells cannot present 258–265 directly because they do not express K^b. Also, when A9-T7 cells are transfected with plasmids encoding polypeptides controlled by the T7 promoter, they fail to express them because the uncapped RNA is very unstable. However, when also infected with vaccinia virus (wild type or otherwise), the RNA is stabilized and they produce a large amount of Ag (12). In our assays, A9-T7 were seeded in six-well plates (4×10^5 cells/well) or 24-well plates (2×10^5 cells/well) incubated overnight at 37°C, and transfected with the indicated plasmids using lipofectamine 2000. For Fig. 3*e*, the cells were transfected for a second time 6 h later. Four hours later, the monolayer was washed twice with PBS and the cells were infected for 1 h with 10 PFU/cell of wild-type WR vaccinia virus in medium containing 2.5% FCS. Next, the monolayer was washed twice with complete medium and incubated in complete RPMI 1640 for 4 h. When required, the proteasome inhibitors lactacystin (2 μM; Sigma-Aldrich) or epoxomicine (160 nM) were added 30 min before the infection.

pAPCs. Bone marrow Mϕ were generated as previously described (16). In our assay, B6 macrophages (Mϕ) can present 258–265 because they express K^b. However, they cannot express the Ag even if they become infected, because they are not transfected and do not express the T7 polymerase.

Determination of cross-presentation. For the standard cross-presentation assay, donor cells were collected, washed five times, resuspended in complete medium, and serially diluted (1/2) in duplicate wells of 96-well plates with a starting concentration of 10^5/well. Mϕ were collected with a rubber policeman, washed, counted, and 10^5 added to each well containing donor cells and incubated for 4 h. For the in situ cross-presentation assay, donor cells that had been infected 8 h earlier were rinsed three times to eliminate detached donor cells, and 4×10^5 Mϕ were added to each well (in Fig. 2*e*, the Mϕ were previously fixed with 0.5% paraformaldehyde and thoroughly washed) and incubated for 4 h. The Mϕ/donor cell mixtures were harvested with a rubber policeman, fixed with paraformaldehyde, and serially diluted (1/2) in duplicate in 96-well plates. B3Z cells (10^5) were added to each well and activation of the hybridoma was determined as before. No cross-presentation was observed when we used as mock donor cells parent A9 cells subjected to identical treatment as donor A9-T7. This type of control was used in every experiment (not shown).

Results and Discussion

We have previously used a novel cross-presentation assay (15) to demonstrate that primary H-2^b Mϕ and DC efficiently cross-present the K^b-restricted epitope 258–265 of chicken OVA (386 aa) acquired from A9-T7 cells at early stages of vaccinia virus infection and expressed within the cytosolic fragment 197–386 (15). However, whether the Ag transferred from donor cells to pAPC is the whole 197–386 translation product, the 258–265 mature epitope, or an intermediate fragment was not explored. To determine whether the mature epitope can be efficiently transferred and cross-presented, we compared the efficiency whereby Mϕ cross-present 197–386 and the mature epitope expressed from a minigene encoding (M)258–265, where (M) is an additional methionine that is required to initiate translation and that is presumably cotranslationally removed. As Ag donor cells, we used vaccinia-infected A9-T7 cells (H-2^k) that had been transfected with the required DNA constructs controlled by the T7 promoter. As pAPC, we used C57BL/6 Mϕ. To detect cross-presentation, we used the B3Z T cell hybridoma that produces β-gal when it specifically recognizes 258–265 bound to H-2K^b. Strikingly, there was no detectable cross-presentation of (M)258–265 while 197–386 was efficiently cross-presented (Fig. 1*a*). The absence of cross-presentation of (M)258–265 was not due to its inefficient expression because (M)258–265 was better than 197–386 in direct presentation assays using as APCs H-2^b MC57G cells infected with recombinant vaccinia-T7. This higher potency of 258–265 in direct presentation was observed when using limiting numbers of APCs (Fig. 1*b*), and across a wide range of DNA used for transfection (Fig. 1*c*) and is expected because 100% of (M)258–265, but only 2.5% of a translated protein, should result in 258–265 (17). Interestingly, we also found that (M)50–386, another truncated version of OVA expected to remain in the cytosol and that is not secreted to the medium (not shown) was cross-presented by Mϕ and presented directly by MC57G cells with very similar efficiency as compared with 197–386. However, full-length OVA, which is mostly secreted (not shown) was presented directly by MC57G cells with somewhat lower efficiency than 197–386 but cross-presented by Mϕ with 80% less efficiency (Fig. 1, *d–f*) probably because it is secreted. Therefore, cross-presentation of cytosolic fragments was efficient, cross-presentation of the secreted full-length OVA was poor, and that of the mature peptide was not detected.

In the previous experiments, (M)258–265 was expressed in the cytosol where short peptides are very unstable (18). To determine whether cross-presentation of the mature epitope could be detected by increasing its chances to bind gp96 or other ER resident chaperones, we expressed 258–265 directly in the ER of donor cells

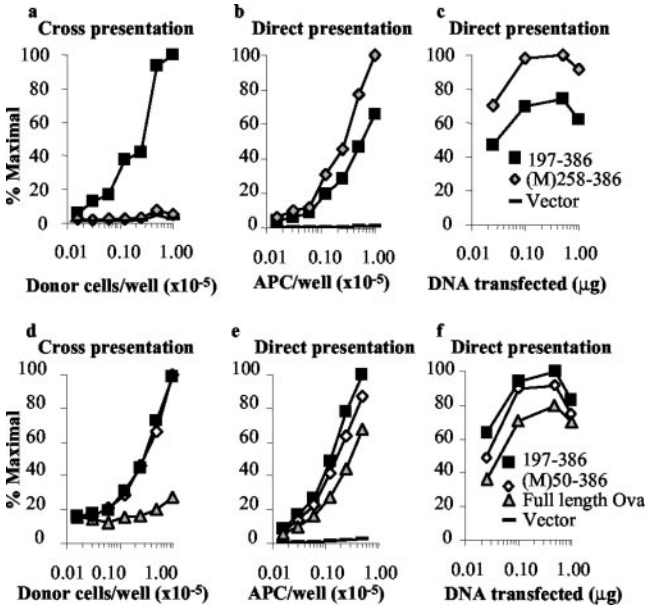

FIGURE 1. The minimal epitope is not cross-presented and the full-length protein is poorly cross-presented. Cross-presentation and direct presentation of the indicated constructs. For cross-presentation, pAPC were C57BL/6 Mϕ and Ag donor cells were A9-T7 cells that were transfected with the indicated constructs and infected with wild-type vaccinia. For direct presentation, APC were MC57G cells transfected with the indicated constructs and infected with recombinant vaccinia-T7. Cross-presentation and direct presentation assays were performed as described in *Materials and Methods*. *b* and *e*, MC57G cells were transfected with 1 μg of plasmid and variable numbers used as APCs in direct presentation experiments. *c* and *f*, MC57G cells were transfected with variable amounts of plasmids and plated at a concentration of 10^5/well.

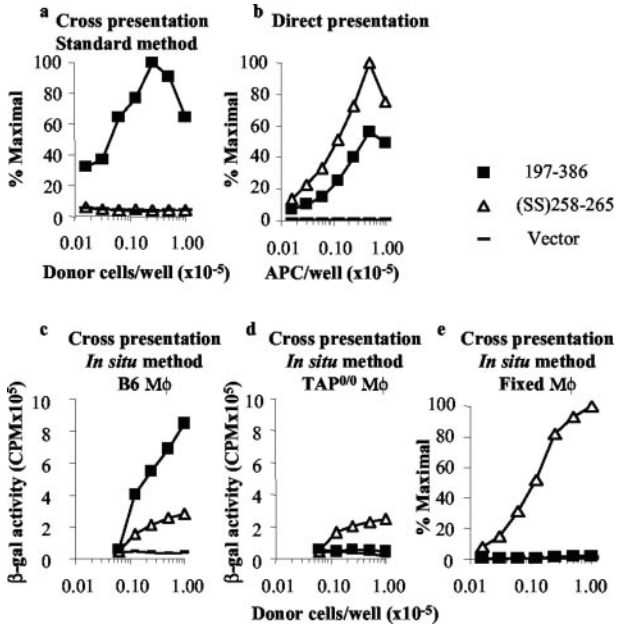

FIGURE 2. The minimal epitope in the ER is inefficiently cross-presented as free secreted peptide binding to surface MHC class I. Cross-presentation and direct presentation of 197–386 and (SS)258–265 with constructs and cells as indicated. The assays were performed as detailed in *Materials and Methods*.

using a plasmid encoding (SS)258–265 where (SS) is the ER insertion/signal sequence derived from the adenovirus E3/19K glycoprotein that is removed cotranslationally (19). However, we did not detect cross-presentation of (SS)258–265 (Fig. 2*a*) even though it was more potent than 197–386 in direct presentation (Fig. 2*b*) further indicating that the dominant form of the transferred Ag is a longer precursor.

In the cross-presentation experiments described thus far, the donor cells were harvested and washed before mixing with the Mϕ. We have also described an alternative "in situ" cross-presentation assay where pAPC were added to the donor cells that remained attached to the plate in which they were originally infected (15). Interestingly, with this in situ assay we were able to detect some cross-presentation of (SS)258–265 perhaps because the method is somewhat more sensitive. Nonetheless, the cross-presentation of (SS)258–265 was much lower than that of 197–386 (Fig. 2*c*). This cross-presentation of the mature epitope was due to direct binding of the peptide to MHC class I molecules at the surface of pAPC because it was cross-presented by $TAP^{0/0}$ Mϕ (Fig. 2*d*) and by Mϕ that had been fixed with paraformaldehyde before being added to the donor cells (Fig. 2*e*). As expected, $TAP^{0/0}$ or previously fixed pAPC did not cross-present 197–386, which we already demonstrated follows the cytosolic route of cross-presentation (15). Therefore, although some cross-presentation of the mature epitope expressed in the ER of donor cells can be observed, it is not the dominant mechanism because it is much lower than the cross-presentation of 197–386. Moreover, this inefficient cross-presentation of the mature epitope is due to peptide binding at the surface of the pAPC (probably because when expressed in the ER, it is secreted as free peptide) and does not follow the HSP model that involves receptor-mediated uptake and internalization.

The data in Figs. 1 and 2 indicates that cross-presentation by transfer of the mature epitope is unlikely to occur under physiological circumstances and that efficient cross-presentation requires the epitope to be embedded within a longer polypeptide. Therefore, our results seemed to indicate that the protein model of cross-presentation was correct. A prediction of the protein model is that blocking the degradation of proteins in donor cells should result in enhanced cross-presentation. Because the proteasome is responsible for most of the cellular protein degradation (20), we determined cross-presentation from donor cells that had been treated with 2 μM lactacystin, a specific inhibitor of the proteasome (21). Contrary to the predictions of the protein model, and to our surprise, lactacystin inhibited cross-presentation of 197–386 (Fig. 3*a*), although some residual cross-presentation was consistently observed (~20%). Similar results were obtained with (M)50–286 and full-length OVA (not shown). It is important to emphasize that we avoided inhibiting the proteasome of the pAPC by thoroughly washing the donor cells (five times) before mixing them with Mϕ. Moreover, we confirmed that lactacystin was not being transferred to Mϕ in experiments in which donor cells previously incubated with up to 16 μM lactacystin did not inhibit direct presentation by Mϕ infected with recombinant vaccinia-OVA (not shown). Importantly, the 2 μM concentration of lactacystin was sufficient to inhibit the direct presentation of 197–386 (Fig. 3*b*) but had no effect in the direct presentation of (M)258–265 (Fig. 3*c*) ruling out a toxic effect of the inhibitor. Furthermore, lactacystin did not affect the expression of 197–386 by donor cells (not shown) as determined by a previously described ELISA (15). In addition, Fig. 3*d* demonstrates that treatment of donor cells with 160 nM epoxomicin, another specific inhibitor of the proteasome (22), also resulted in strong inhibition of 197–386 cross-presentation. From these experiments, we conclude that the dominant mechanism of cross-presentation is the transfer of proteasome intermediates.

The results above demonstrate that the majority of the transferred Ag responsible for cross-presentation is a proteasomal intermediate and not the mature epitope or the intact translation

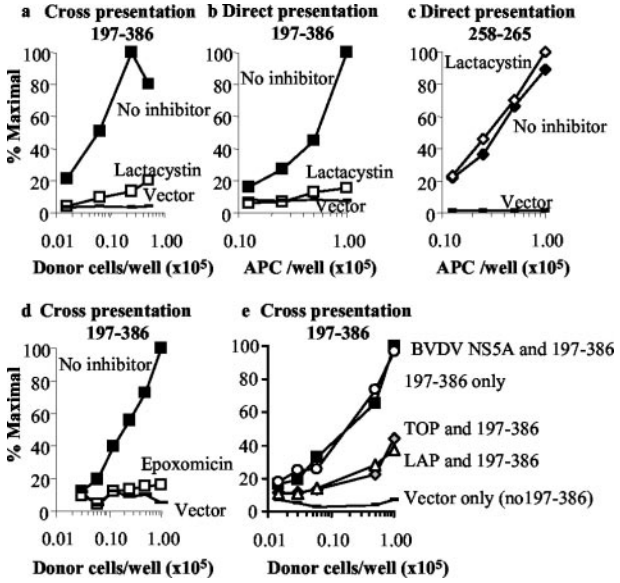

FIGURE 3. The bulk of a cross-presented Ag is a product of the proteasome and sensitive to cytosolic proteases. Cross-presentation or direct presentation of 197–386 and 258–265 in the absence or in the presence of the proteasome inhibitors lactacystin or epoxomicin as indicated. *e*, The Ag donor cells overexpressed the cytosolic peptidases TOP or LAP or the irrelevant control BVDV NS5A. Black lines represent cells transfected with vector instead of 197–386. Assays were performed as detailed in *Materials and Methods*.

product. Because most products of the mammalian proteasome range from 5 to 22 aa (23), we speculated that the dominant form of the transferred Ag is an oligopeptide longer than the mature epitope. To determine whether this is the case and whether the putative oligopeptides are free in the cytosol of the donor cell and susceptible to proteases, we tested whether cross-presentation of 197–386 could be inhibited by the overexpression of two peptidases that were previously shown to be involved in MHC class I Ag presentation: TOP (a metalloendopeptidase that only digests oligopeptides) and LAP (24–27). For this purpose, we used Ag donor cells that had previously been transfected with the plasmid pcDNA (Invitrogen) encoding TOP, LAP, or a completely irrelevant protein (NS5A from bovine viral diarrhea virus) that we had available in the appropriate vector and that is not toxic to cells. Cross-presentation was determined as usual. Strikingly, overexpression of both peptidases but not of control NS5A inhibited cross-presentation (Fig. 3*e*). This indicates that the bulk of transferred Ags are oligopeptides because they are susceptible to TOP digestion. Moreover, the data indicated that at some point in time, the Ag precursors must be free in the cytosol and unprotected by chaperones because they are susceptible to the action of both cytosolic peptidases.

An important observation stemming from our experiments is that cross-presentation of the model Ag OVA expressed by donor cells at early stages of vaccinia infection cannot be attributed to the transfer of the mature epitope either free or associated with chaperones. We reach this conclusion because we observed efficient cross-presentation of the longer precursors 197–386 and (M)50–386 but not of the mature epitope (M)258–265 even though the molar concentration of mature epitope must be higher when expressing the minigenes than when expressing the longer precursor (17) and is inferred from experiments where the direct presentation of (M)258–265 and (SS)258–265 was more efficient than that of 197–386. Of note, we were able to observe some cross-presentation of the mature epitope expressed in the ER using an alternative cross-presentation assay. However, in physiological situations this type of cross-presentation is probably not important because it requires the expression of large amounts of peptide in the ER. Nevertheless, because fixed pAPC were able to cross-present (SS)258–265, we can conclude that the uptake and internalization of the 258–265 mature epitope bound to gp96 or other ER chaperones through CD91 or other cell surface receptor is not responsible for the cross-presentation that we observed. In addition, our experiments demonstrate that relatively long cytosolic fragments are much more potent at cross-presentation than the full-length protein which is secreted. This is consistent with our previous demonstration that cross-presentation requires donor cell-pAPC contact (15).

For those with an inclination toward the protein model, an unanticipated finding of this report is that the bulk of the cross-presented Ag is a product of the donor cell proteasome and not the intact translation product. However, this finding does not rule out that the transfer of protein could play a role in cross-presentation and cross-priming. Indeed, we also observed ~20% lactacystin-resistant cross-presentation when the Ag donors were UV irradiated cells that had been infected with recombinant vaccinia-OVA (our unpublished results). However, the use of these types of donors result in a much larger signal for both the lactacystin-sensitive and lactacystin-resistant cross-presentation. Therefore, under conditions of saturating amounts of Ag, the lactacystin-sensitive cross-presentation may not be readily apparent. This may be particularly relevant in vivo, where the parameters of $CD8^+$ T cell activation may not be absolutely quantitative but rather dependent on thresholds. Nonetheless, our data clearly establishes that the transfer of the intact protein is not the major mechanism of cross-presentation when Ag is acquired from donor cells at an early phase of vaccinia infection. Certainly, experiments with other model Ags will be required to confirm the general validity of our present observations.

An emerging task is to define the mechanism whereby proteasomal products are transferred. One possibility is that these products are transferred as free oligopeptides. This could follow the same mechanism as for undigested proteins or could be mediated by a mechanism that preferentially transfers oligopeptides from the endocytic vacuoles of pAPC to their cytosol. Alternatively, the products of the proteasome could bind to chaperones in a slightly modified version of the HSP model. If this second model is correct, the binding of the oligopeptides to the HSP might occur by diffusion from the proteasome to the HSP because our experiments with cytosolic peptidases indicate that there is a particular stage when the cross-presented material is free in the cytosol. An interesting teleological question is why proteasomal products are preferred over intact proteins. Because newly synthesized defective proteins constitute a large fraction of the proteasome substrate (28), it is possible that in acute infections where most efforts are directed toward the synthesis of viral proteins, the use of proteasome products tilts the balance in favor of the cross-presentation of viral proteins over that of competing cellular proteins.

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