The Exception that Reinforces the Rule: Crosspriming by Cytosolic Peptides that Escape Degradation

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SUMMARY

The nature of crosspriming immunogens for CD8+ T cell responses is highly controversial. By using a panel of T cell receptor-like antibodies specific for viral peptides bound to mouse Dβ major histocompatibility complex class I molecules, we show that an exceptional peptide (PA224-233) expressed as a viral minigene product formed a sizeable cytosolic pool continuously presented for hours after protein synthesis was inhibited. PA224-233 pool formation required active cytosolic heat-shock protein 90 but not ER g96 and uniquely enabled crosspriming by this peptide. These findings demonstrate that exceptional class I binding oligopeptides that escape proteolytic degradation are potent crosspriming agents. Thus, the feeble immunogenicity of natural proteasome products in crosspriming can be attributed to their evanescence in donor cells and not an absolute inability of cytosolic oligopeptides to be transferred to and presented by professional antigen-presenting cells.

INTRODUCTION

Direct antigen presentation is based on proteasome-initiated degradation of defective ribosomal products (DRiPs) and other rapidly degraded endogenous translation products (Eisenlohr et al., 2007; Yewdell, 2007). Typically, peptides of eight or more residues are transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER) where final trimming of COOH-terminal residues is mediated by ER-associated aminopeptidase (ERAAP). Peptide binding releases nascent class I molecules from the loading complex, initiating their transport to the plasma membrane for recognition by CD8+ T cells.

In viral infections, direct presentation enables CD8+ T cell activation by virus-infected cells, including dendritic cells (DCs), which can initiate naïve CD8+ T cell response in lymph nodes and spleen. But what if viruses cannot (or will not) express their proteins in DCs? In this case, an alternative pathway exists, termed crosspriming, where DCs process antigens acquired from infected cells and present them to naïve CD8+ T cells (Shen and Rock, 2006). Controversy swirls around virtually every aspect of crosspriming.

A substantial literature has grown around the concept that crosspriming is mediated by DC acquisition of glucose-regulated protein 94 (GRP94) (also known as glycoprotein 96 [gp96]) and other chaperones bearing oligopeptides derived from proteasomal antigen degradation (Srivastava, 2002). It has proven difficult, however, for many laboratories to recover peptides bound to gp96 purified from antigen-presenting cells, or even to demonstrate that gp96 binds to oligopeptides with an affinity consistent with its proposed role in crosspriming (Demine and Walden, 2005; Javid et al., 2007; Nicchitta et al., 2004; Wallin et al., 2002; Ying and Flatmark, 2006). Purified gp96 and a variety of other chaperones demonstrate antigen-specific crosspriming activity, but this activity is weak, and the adjuvant role of contaminants (e.g., lectins, bacterial lipopolysaccharide) in the phenomenon is a concern. Heat-shock protein 90 (HSP90) has been recovered from cells bound to truncated forms of a model antigen (Kunisawa and Shastri, 2006), but the crosspriming activity of such complexes has not been demonstrated in the context of intact cells as immunogens. Moreover, seven laboratories recently published studies pointing to proteasome substrates rather than proteasome products as the source of crosspriming antigens (Basta et al., 2005; Donohue et al., 2006; Freigang et al., 2007; Gasteiger et al., 2007; Norbury et al., 2004; Shen and Rock, 2004; Wolkers et al., 2004). This conclusion jibes with the original observation by Rammensee and colleagues that antigenic oligopeptides are recovered only from cells expressing class I molecules capable of presenting the peptides (Falk et al., 1990). Further, the finding that cytosolic
oligopeptides are rapidly destroyed by endopeptidases and aminopeptidases (half-life on the order of 10 s) (Reits et al., 2004) is inconsistent with the hypothesis that oligopeptides exist in stable complexes with molecular chaperones.

A key advance in the antigen presentation field was the introduction of antibodies with TCR-like specificities, generated by standard hybridoma technology or by screening of antibody phage display libraries (Andersen et al., 1996; Denkberg and Reiter, 2006; Porgador et al., 1997). Such reagents enable measurement of cell-surface major histocompatibility complex (MHC) class I peptide complexes with great precision by flow cytometry. Here we describe the generation and characterization of a panel of TCR-like phage-displayed antibodies specific for well-defined influenza A virus (IAV) peptides bound to H-2Db molecules. We then use these phage antibodies to study the cell biology and immunology of oligopeptides introduced into the cytosol and ER of antigen-presenting cell and crosspriming donor cells as biosynthesized minigene products.

RESULTS

Isolation of Human Recombinant Antibodies with TCR-like Specificity to Influenza Virus-Derived Peptide-MHC Complexes

To generate a panel of TCR-like antibodies specific for IAV-Db-restricted determinants, we selected a large nonimmune library consisting of 3.7 × 10¹⁰ unique human recombinant phage Fab antibody fragments (de Haard et al., 1999) for binding to purified H-2Db complexed with PA224-233, NP366-374, or PB1-F262-70. These determinants top the defined anti-IAV immunodominance hierarchy in B6 mice. Out of numerous candidates identified, we chose the Fab that demonstrated the best signal-to-noise ratio and sensitivity. As seen in Figure S1 available online, the three Fabs chosen selectively recognized both purified Db and cell-surface Db complexed with their cognate determinants. The threshold for peptide recognition on peptide-pulsed EL4 cells with low-temperature-induced peptide-receptive Db molecules occurred at ~10⁻⁹ M. This is similar to the sensitivity of the 25-D1.16 mAb, which is specific for Kb-SIINFEKL complexes. This reagent represents “gold standard” for highly sensitive and specific TCR-like antibody recognition (Porgador et al., 1997).

We next tested the ability of the TCR-like Fabs (FabTCRs) to bind to endogenous complexes generated from cytosolic or ER-targeted minigene products expressed by recombinant vaccinia viruses (rVV) (see schematic of rVV in Figure S2). Minigene products are typically expressed at very high copy numbers (tens of thousands per cell) relative to peptides generated from full-length proteins (ten to a few thousand copies per cell) (Princiotta et al., 2003). Each of the six minigene products tested was easily detected by its cognate FabTCR (Figure 1). Addition of a signal sequence increased the numbers of cell-surface complexes very slightly for PB1-F262-70 and ~5-fold for NP366-374 and PA224-233.

To determine the TAP dependence of complex formation, we infected human T2-Db cells with the rVV (T2 cells are EBV-transformed lymphoblasts that lack both TAP1 and TAP2 genes, and
consequently, cytosolic peptides have limited access to the ER). This revealed that, as expected, each of the cytosolic minigene products demonstrated only slight TAP-independent presentation, whereas ER targeting bypassed the requirement for TAP for PB1-F262-70 and PA224-233. Surprisingly, presentation of ER-targeted NP366-374 remained largely TAP dependent. A possible explanation is the presence of Ala as the NH2-terminal residue in NP366-374, because Ala is a preferred residue at the P1 site of signal peptidase cleavage, which may therefore destroy the peptide when it is translocated via the translocon. If this is the case, the enhanced ability of ES-NP366-374 to access Dp in L-Dp cells would presumably be due to the ability of the leader sequence to enhance TAP-mediated antigen presentation. This could be due to resistance to degradation by cytosolic or ER proteases or enhanced TAP-mediated transport into the ER of the full-length ES peptide or, more likely, a NH2-terminally trimmed version.

These findings demonstrate that Fab TCRs can be used as highly specific reagents to precisely quantitate cell-surface Dp-antigen complexes generated from VV-encoded minigenes.

Kinetics of Cell-Surface Complex Presentation via TCR-like Fabs

We next examined the kinetics of cell-surface complex expression at 30 min intervals after infection of L-Dp cells with rVVs expressing the three determinants. In each case, cognate peptide-MHC complexes were first detected by the FabTCRs 60–90 min after infection and quickly reached V_{max} of complex generation (i.e., the maximal increase in the rate of complex accumulation). This behavior is similar to expression of K0-Ova257-264 complexes (Princiotta et al., 2003) and is exactly what is expected for peptides that rapidly achieve saturating amounts of expression for feeding the class I pathway and require little or no processing for binding to class I molecules.

Addition of BFA, which blocks MHC egress from the secretory pathway by inducing Golgi complex disassembly and redistribution of Golgi complex proteins into the ER (Yewdell and Bennink, 1989), resulted in an immediate block of cell-surface complex accretion. In the case of ES-PB1-F262-70, BFA caused a steep and steady decline in surface complexes, indicative of a relatively low complex stability (t_{1/2} of ~120 min). Under the same conditions, both ER-targeted and cytosolic NP366-374 demonstrated intermediate complex stability whereas both cytosolic and ER-targeted PA224-233 generated highly stable complexes. This hierarchy of complex stability agrees with the stability of complexes generated with synthetic peptides that we previously reported (Figure 3 in Chen et al. [2004]), findings that we repeated by using the FabTCRs (data not shown).

Cellular oligopeptides are believed to be nearly completely degraded in a few seconds (Reits et al., 2004), so the kinetic difference between blocking protein synthesis and blocking surface delivery with BFA should be minimal and difficult to detect when sampling cells at 30 min intervals. This was indeed the case for NP366-374-Dp and PB1-F262-70-Dp complexes (Figures 2B, 2C, 2E, and 2F). Dp-PA224-233 complexes behaved in a completely different and highly surprising manner. The delivery of PA224-233 complexes to the cell surface continued at nearly the same rate up to 2 hr after addition of protein synthesis inhibitors (PSI) (Figures 2A and 2D).

Figure 2. Kinetics of Dp-Peptide Presentation on L-Dp Cells after rVV Infections

Cell-surface Dp-peptide amounts were measured on L-Dp cells infected with rVVs expressing ES-224 (A), ES-366 (B), ES-PB1-F2 (C), Mg-224 (D), Mg-366 (E), or Mg-PB1-F2 (F). Infected cells were treated with protein synthesis inhibitors (PSI) or BFA at times indicated by arrows. In (G), PSI were added to VV-ES-224-infected cells at the times indicated, and cell-surface Dp-PA224-233 amounts were determined. Addition of PSI at 150 min and 180 min reveals PA224-233 pools that result in increases of 70 and 105 fluorescence units of Dp-PA224-233 complexes, respectively. Each experiment was repeated at least three times.
A trivial explanation for this finding is that the translation of PA224-233 is resistant to the protein synthesis inhibitors we utilized. Based on the mode of action of protein synthesis inhibitors, we considered this very unlikely (particularly because a number of potent protein synthesis inhibitors [anisomycin, cycloheximide, emetine, pactamycin] used individually gave similar results). We eliminated this possibility by treating cells with protein synthesis inhibitors at the start of infection and showing that D<sup>3</sup>-PA224-233 complex formation was completely inhibited (not shown). Rather, these findings demonstrate the formation of a pool of PA224-233 peptide or D<sup>3</sup>-PA224-233 complexes that feeds the class I pathway for hours after protein synthesis shutdown.

The kinetics of pool formation is most consistent with the former possibility. When we blocked protein synthesis at increasing times after infection, the size of the pool increased with time of expression. In the interval between 150 and 180 min, the pool increased from 70 flow units to 105 units (Figure 2G). Notably, this is twice the amount of complexes delivered to the cell surface over the identical interval. Because the V<sub>max</sub> of cell-surface complex delivery has been reached by 150 min, the large increase in the size of the pool over the next 30 min is difficult to reconcile with a pool of D<sup>3</sup>-PA224-233 complexes in the secretory pathway if the size of this pool is directly related to the rate of ER export and cell-surface complex delivery.

These experiments demonstrate that PB1-F2<sub>62-70</sub> and NP<sub>366-374</sub> demonstrate kinetic behavior completely consistent with rapid degradation unless bound to MHC class I molecules, whereas PA224-233 demonstrates kinetics not previously observed (for either proteins or minigene products) that are consistent with it forming a substantial pool that forms a steady source of peptides for hours after shutting off its biosynthetic source.

**PA224-233 Peptide Cellular Pool Is Dependent on Cellular HSP90 Activity**

The recent report from Kunisawa and Shastri (2006) demonstrating that Hsp90α associates with proteolytic fragments of KOVAK, a misfolded cytosolic model antigen, prompted us to examine the effect of cell-permeant small molecule inhibitors of HSP90. These inhibitors are among the most selective inhibitors available to probe intracellular pathways. All of the inhibitors used target the NH<sub>2</sub>-terminal ATP binding domain of HSP90 family members. Two of the inhibitors used, 17AAG and 17DMAG, are second generation geldanamycin analogs with higher potency than the parent, while the third, radicicol, is chemically distinct from geldanamycin, and so would be expected to have a distinct off-target profile. Cells were treated with the given HSP90 inhibitor at 30 min after infection to allow viral penetration to occur and to minimize effects on viral gene expression (Figure 3). Treatment with each of the inhibitors inhibited D<sup>3</sup>-PA224-233 cell-surface complex expression by approximately 20%–30%. This is not specific for PA224-233, as shown by the fact that NP366-374 and PB1-F2<sub>62-70</sub> decreased similarly (data not shown) and probably reflects a requirement for active HSP90 for maximal VV gene expression.

To assess the effect of HSP90 inhibitors on PA224-233 pool formation in this experiment, we incubated the cell with protein synthesis inhibitors at 240 min (Figures 3E–3G). This revealed that each of the HSP90 inhibitors tested eliminated the PA224-233 peptide pool. Importantly, addition of HSP90 inhibitors simulta-
FRAP evidence for the physical interaction of a HSP90 family member peptide binding site. This contrasts with HSP90\(\beta\), whose mobility is reported to be insensitive to HSP-90 inhibitors (Picard et al., 2006).

These data demonstrate that 17AAG detectably affects HSP90 mobility in living cells and are consistent with the conclusion that PA 224-233 associates with a cytosolic partner that requires HSP90 peptide binding activity: either HSP90 itself, or a client that is rather rapidly altered by HSP90 inhibition.

**PA\textsubscript{224-233} Peptide Minigene Crossprimes CD8\textsuperscript{+} T Cells in B6 Mice**

The observation that PA\textsubscript{224-233} accumulates in a substantial stable pool in L-D\(^\text{0}\) cells prompted the question of whether the pool can be tapped for crosspriming in vivo. To address this question, we immunized B6 mice via intraperitoneal (i.p.) injection with rVV-infected L929 cells (i.e., the original nontransfected cells that do not express D\(^\text{0}\)) that were irradiated with UV light to destroy residual viral infectivity. We determined the frequency of local (peritoneal exudate cells [PEC]) and splenic responding CD8\textsuperscript{+} T cells by intracellular staining for INF-\(\gamma\) after incubation of antigen-presenting cells (APCs) with peptides corresponding to the cognate minigene product or to a peptide corresponding to residues B8R\textsubscript{20-27}, the immunodominant determinant in VV infections in B6 mice. For an estimate of overall anti-VV responses, we also stimulated responding CD8\textsuperscript{+} T cells ex vivo with VV-infected DC2.4 or L-D\(^\text{0}\) cells.

The data in Figure 6A clearly show that L929 cells expressing NP\textsubscript{366-374} and PB1-F\textsubscript{262-70} as ER-targeted minigene products fail to elicit CD8\textsuperscript{+} T cells responses that are substantially above background values obtained by stimulating with an irrelevant peptide. By contrast, cells expressing ER-targeted PA\textsubscript{224-233} elicited robust PA\textsubscript{224-233}-specific responses (Figures 6A and 6B). Note that cells expressing the nonimmunogenic minigene products elicited vigorous B8R\textsubscript{20-27} and total VV-specific CD8\textsuperscript{+} T cell responses, indicating that the nonimmunogenicity of their minigene products cannot be attributed to diminished infectivity or other factors that limit the crosspriming capacity of these rVVs.

To determine the TAP dependence of crosspriming, we performed a similar experiment in which we replaced L929 cells with T2 cells (again lacking D\(^\text{0}\)). Both cytosolic and ER-targeted PA\textsubscript{224-233} minigenes crossprimed to a similar extent for PA\textsubscript{224-233}-specific responses (Figure 6B). (Note that it is likely that substantial amounts of ER-targeted peptides persist in the cytosol, resulting either from inefficient targeting of such small proteins to the ER or from the recycling of excess peptides from the ER).
to the cytosol [Koopmann et al., 2000; Roelse et al., 1994]. The TAP independence of cytosolic PA224-233 crosspriming is in agreement with the finding that cytosolic HSP90 but not GRP94 is involved in peptide pool formation (Figure 4). We more directly tested the involvement of functional HSP90 in PA224-233 crosspriming by treating donor cells with 17AAG during rVV infection prior to their introduction into B6 mice (Figure 6C). This reduced PEC and splenic anti-PA224-233 responses by ~85% while reducing B8R20-27 or overall VV-specific response by 15% to 50%.

Next, we examined the requirement for GRP94 in crosspriming by using human HEK293 cells stably transfected with GRP94-targeting siRNA (control HEK293 cells were stably transfected with the same sequence but scrambled). Via immunoblotting, GRP94 is undetectable in knockdown cells whereas HSP90 amounts are similar between control and knockdown cells (Figure 6D). Despite the absence of GRP94, both rVVs expressing cytosolic and ER-targeted PA224-233 were effective at crosspriming for PA224-233-specific, B8R20-27-specific, and total VV-specific CD8+ T cell responses (Figures 6E and 6F).

**Crosspriming Is Based on the Presence of a Large D8-Independent Pool**

To characterize the crosspriming pool of PA224-233 in L929 cells, we measured antigenic peptides recovered from HPLC-fractionated low-molecular-weight acid-soluble material from homogenized cells infected with VV-encoded cytosolic PA224-233 or (as a negative control) NP366-374. Activity was measured with CD8+ T cell populations expanded in vitro to each peptide. T cell populations demonstrated highly similar picomolar sensitivity to synthetic peptide (data not shown). Large amounts of NP366-374 were recovered from infected L-D8 cells, with recovery of peak antigenic activity in fractions that coelute with synthetic NP366-374 (Figure 7A). Antigenic activity in peak fractions could be detected...
Peptide-Based Crosspriming

Proteasomal protein cleavage generates oligopeptides of between 3 and 32 residues that are degraded by highly active cytosolic endopeptidases and aminopeptidases (Kisselev et al., 1998; Reits et al., 2004). The half-life of oligopeptides in this aggressively degradative environment is believed to be ~10 s. Inasmuch as proteasome-generated peptides are functionless, potentially dangerous agents that can interfere with cellular functions (including impeding the interaction of molecular chaperones with their substrates), the rapid removal of peptides and reutilization of amino acids is logical.

Despite this, a considerable literature has accrued regarding the potential role of GRP94 and other abundant molecular chaperones in protecting peptides from degradation and promoting peptide immunogenicity upon transfer to pAPCs. There is compelling evidence that molecular chaperones have a bona fide role in activating innate immune receptors (Baker-LePain et al., 2002; Oizumi et al., 2007). The function of molecular chaperones, however, in specifically binding and delivering oligopeptides generated from biosynthesized proteins to pAPCs is controversial. Molecular chaperones exhibit low affinity for their substrates (on the order of 0.1 mM), consistent with their need to transiently interact with clients to promote folding (Flynn et al., 1989, 1991; Fourie et al., 1994; Ying and Flatmark, 2006). With such a low affinity, peptides would be protected only fleetingly, achieve low steady-state amounts in living cells, and dissociate rapidly upon release from cells and delivery to pAPCs. Although there is limited evidence for recovery of defined peptides from GRP94 purified from cells, others have found only minute quantities of peptides bound to GRP94 and have questioned the peptide-chaperoning function of GRP94 in antigen presentation (Demine and Walden, 2005; Nicchitta et al., 2004; Wallin et al., 2002; Ying and Flatmark, 2006). Moreover, molecular chaperones are only weakly immunogenic relative to cell-associated immunogens, even when loaded in vitro to the maximal extent attainable with synthetic peptides. Even non-cell-associated heat-aggregated viruses or proteins are more immunogenic than the most potent HSP preparations reported (Cho et al., 2003; Speidel et al., 1997).

On top of this, we and others have provided evidence strongly supporting a central role for intact proteins in crosspriming (Basta et al., 2005; Donohue et al., 2006; Freigang et al., 2007; Gasteiger et al., 2007; Norbury et al., 2004; Shen and Rock, 2008).
2004; Wolkers et al., 2004). Cellular oligopeptides generated via signal peptidase liberation or synthesized as cytosolic or ER-targeted minigene products display little to no crosspriming activity (Gasteiger et al., 2007; Norbury et al., 2004; Serna et al., 2003). The poor crosspriming capacity of minigene products, consistent with the lack of crosspresentation of minigene products observed in vitro by Serna et al. (2003), can be explained by their rapid degradation. Like naturally processed antigenic peptides, recovery of minigene-encoded minimal peptides from cells is heavily dependent on the presence of a high-affinity class I receptor (Anton et al., 1997). More recently, we precisely quantitated cell-surface K\(^\alpha\)-Ova\(^{257-264}\) complex expression in cells infected with VV-Ova\(^{257-264}\) by using the TCR-like mAb 25-D1.16 (Princiotta et al., 2003). Upon abrogation of protein synthesis, delivery of K\(^\alpha\)-Ova\(^{257-264}\) complexes to the cell surface ceased rapidly, consistent with the rapid degradation of Ova\(^{257-264}\).

In the present study, we extend these findings to three additional defined peptides expressed as cytosolic or ER-targeted minigenes. By using a novel panel of TCR-like Abs to measure cell-surface D\(^\beta\)-peptide complex expression, we found that for two of the peptides, NP\(^{366-374}\) and PB1-F2\(^{62-70}\), the kinetics of shutdown of complex delivery to the cell surface after inhibiting protein synthesis was virtually identical to those observed after BFA blockade of D\(^\beta\)-peptide complex egress from the early...
secretory pathway, providing unequivocal evidence for the absence of a protected peptide pool capable of supplying the class I pathway. Crucially, these peptides also failed to exhibit substantial crosspriming activity when expressed in cells lacking D\(^\text{E}\).

In distinct contrast, whereas BFA rapidly abrogated cell-surface delivery of PA\(_{224-233}\) complexes, delivery continued for hours after abrogation of protein synthesis, indicative of a sizable intracellular pool. This pool is independent of D\(^\text{E}\) expression, as demonstrated by the crosspriming activity of PA\(_{224-233}\) minigene products in L929 cells and also the recovery of large amounts of PA\(_{224-233}\) via acid extraction from the same cells. The TAP independence of PA\(_{224-233}\) minigene product crosspriming points to a cytosolic location for the PA\(_{224-233}\) pool. Consistent with this interpretation, the PA\(_{224-233}\) pool and its crosspriming activity is abrogated by selectively interfering with cytosolic HSP90 function or expression and is unaffected by ER GRP94 knockdown. Further, we show that upon microinjection, a fluorescent derivative of PA\(_{224-233}\) diffuses more slowly in the cytosol than an equivalent form of PB1-F2\(_{62-70}\), an effect that is rapidly abrogated by addition of a HSP90 inhibitor. This is consistent with the binding of PA\(_{224-233}\) to a HSP90-dependent cytosolic partner that retards its diffusion.

We were unable to recover PA\(_{224-233}\) with HSP90-specific antibodies to purify HSP90 from cytosol recovered from semi-intact cells or TX100 lysates (data not shown). Kunisawa and Shastri (2006) recovered numerous fragments of KOVAK from HSP90 antibody pulldowns. It is uncertain, however, whether these peptides directly associate with HSP90, because HSP90 participates in large complexes containing other client-binding molecular chaperones. Two findings favor the idea that PA\(_{224-233}\) is only indirectly influenced by HSP90 activity. First, the PA\(_{224-233}\) pool persists for hours after protein synthesis inhibition, suggesting a high-affinity interaction with its chaperone. Second, adding HSP90 inhibitors at the time of protein synthesis blockade did not deplete the pre-existing PA\(_{224-233}\) pool. This strongly suggests that pool formation is not directly based on association of PA\(_{224-233}\) with HSP90, because HSP90 inhibitors would be predicted to cause either the release of PA\(_{224-233}\) from HSP90 and rapid proteolysis or the Ub-dependent degradation of HSP90-bound PA\(_{224-233}\) (Isaacs et al., 2003). In support of the rapid action of HSP90 inhibitors, we show that 17AAG rapidly increases the diffusion of HSP90\(\text{a}\) in living cells.

Notably, inactivation or depletion of HSP90 and GRP94 had little effect on the crosspriming capacity of BBR\(_{20-27}\)-specific CD8\(^{+}\) T cells or polyclonal anti-VV CD8\(^{+}\) T cell responses. It is possible that other molecular chaperones are involved in crosspriming for these natural gene products. We believe it is unlikely, however, that this involves chaperoned peptides generated from these proteins.

Our findings demonstrate that oligopeptides are capable of crosspriming if they are spared from proteolysis. Thus, the inability of other minigene products or efficiently liberated peptides (such as signal sequences or oligopeptides released from Ub-fusion proteins by Ub-hydrolases) to crossprime can be directly attributed to their evanescence and not the general inability of oligopeptides to survive transfer to, and further processing in, pAPCs. This strongly implies that the poor immunogenicity of rapidly degraded model proteins, and presumably natural DR\(\text{a}\)s as well, can be attributed to the rapid degradation of most pro-semaonal products, which simply do not achieve the steady-state quantities needed for crosspriming. On the other hand, our finding that a subset of peptides is resistant to rapid proteolysis would favor the possible contribution of CD8\(^{+}\) T cell priming via oligopeptide transfer to pAPCs through gap junctions, which has been elegantly shown to function cultured cells by Neefjes and colleagues (Neijssen et al., 2005, 2007).

**EXPERIMENTAL PROCEDURES**

**Mice**

All experiments used 6- to 10-week-old female C57BL/6J mice purchased from Taconic Farms. Mice were housed in the animal care facility at National Institute of Allergy and Infectious Diseases (NIAID) under specific pathogen-free conditions and maintained on standard mouse chow and water provided ad libitum. The NIAID Animal Care and Use Committee approved all animal procedures and studies.
Peptides, MHC Complexes, and Reagents
Peptides were procured and characterized by the Biologic Resource Branch, NIAID (Rockville, MD). In each case, substances with the predicted mass content constituted >95% of the material analyzed. Peptides were dissolved in DMSO at 10 mM and stored at -20 °C. FITC-labeled peptides (LSLRNFLPVK-c-fluorescein)-OH, PA224-233, or SSLLENFRAYK-c-fluorescein)-OH, PB1-F2αβ-70 were dissolved in DMSO at a concentration of 10 μg/μl and kept in -20 °C until use. Immediately before microinjection, stock peptide solution was diluted to 100 μg/ml in Dulbecco’s PBS (pH 7.4) with calcium and magnesium (DPBS). Brefeldin A (BFA) (Sigma, St. Louis, MO) was added to a final concentration of 5 μg/ml purified Fab antibody. Cells were then washed and incubated for 1 h at RT with 0.25 μg/ml of MHC-peptide complexes. The plates were blocked for 30 min at RT with PBS/2% skim milk and then incubated for 1 h at RT with phage clones (~10^9 phages/well) or various concentrations of soluble purified Fab. After washing, the plates were incubated with HRP-conjugated/anti-human Fab antibody (for soluble Fab) or HRP-conjugated anti-M13 phage (for phage-displayed Fab). Detection was performed with tetramethylbenzidine reagent (Sigma-Aldrich, St. Louis, MO).

Expression and Purification of Soluble Recombinant Fab Abs
Fab antibodies were expressed and purified as described (Lev et al., 2002). BL21 cells were grown to OD600 = 1.0 and induced to express the recombinant Fab antibody by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) for 3 h at 30 °C. Proteins were extracted with B-PER solution (Pierce, Rockford, IL) and applied onto a prewashed TALON column (BD, Palo Alto, CA), and bound Fab were eluted with 0.5 mL of 100 mM imidazole in PBS. The eluted Fab were dialyzed against PBS (overnight, 4 °C) to remove residual imidazole.

ELISA with Phage Clones and Purified Fab Antibodies
The binding specificity of individual phage clones and soluble Fab were determined by ELISA with biotinylated MHC-peptide complexes. ELISA plates (Falcon) were coated 1 h at 37 °C with BSA-biotin (1 μg/well). The plates were washed and incubated (O.N. at 4 °C) with streptavidin (1 μg/well), washed extensively, and further incubated (1 h, RT) with 0.25 μg/ml of MHC-peptide complexes. The plates were blocked for 30 min at RT with PBS/2%/skim milk and subsequently were incubated for 1 h at RT with phage clones (~10^9 phages/well) or various concentrations of soluble purified Fab. After washing, the plates were incubated with HRP-conjugated/anti-human Fab antibody (for soluble Fab) or HRP-conjugated anti-M13 phage (for phage-displayed Fab). Detection was performed with tetramethylbenzidine reagent (Sigma-Aldrich, St. Louis, MO).

Flow Cytometry
D^β-peptide amounts were determined by incubating cells for 30 min on ice with 20 μg/ml purified Fab antibody. Cells were then washed and incubated for 20 min with Cy5-conjugated anti-human antibody. Cellular Cy5 amounts were determined on a FACScalibur cytometer (Beckton Dickinson, San Jose, CA) with CellQuest (Beckton Dickinson) software or LSR II (Beckton Dickinson, San Jose, CA) with FACSDiva (BD) software, and data were analyzed with FlowJo (Tree Star, San Carlos, CA) software.

Microscopy and FRAP Analysis
Cells were examined and manipulated with a Leica TCS-SP5 DMi6000 with 8000 Hz resonant scanner and 40 x 1.25 NA oil objective (Leica Microsystems, Germany). The microscope was equipped with a Cube & the Box environmental chamber (Life Imaging Services, Switzerland). Cells were maintained at 37 °C in 95%/5% air/CO2 mixture. Peptides were microinjected into cells with an Eppendorf FemtoJet microinjector system (Eppendorf, NY). Cells were also transfected with eGFP or mCherry-HSP90 plasmids as controls with Amaxa nucleofactor with T-24 program. Prior to FRAP, a rectangular 9 μm x 35 μm area was positioned on a microinjected cell with a bleaching area of 6 μm x 13 μm size placed in the center of the cells. 20 frames in 0.01 s were collected before bleaching was performed for 0.2 s at the maximum laser power of 458, 476, and 488 nm laser lines. Fluorescence recovery was monitored at a collection rate of 100 frames per 0.01 s. At least 50 cells for each condition were measured in more than three independent experiments. FRAP recovery half-times were calculated with Leica Application Suite Advanced Fluorescence software.

Extracting Antigenic Peptides from rVV Minigene-Infected Cells, HPLC, Run, and Measure of CTL Responses
5 x 10^5 L929 and 1.25 x 10^6 L-D^β cells were infected with rVV-PA224-233 or rVV-PA224-233 at 20 pfu/cell. Cells were harvested 3 h after infection, snap frozen, and resuspended in 8 ml 0.1% TFA/H2O. After adding 3 ml of 1% TFA/H2O, cells were disrupted by >20 strokes in a Dounce homogenizer, sonicated,
and rotated on wheel for 30 min at 4 °C. The lysate was pelleted by low-speed centrifugation, and the supernatant was passed through a 0.45 μm syringe filter and then a 3K cut-off filter (Macrosep, filtron 3K, Cat OD003C36, Pall Filtron Corp., Northborough, MA). The filtrate was speed dried with vacuum to a volume of less than 400 μl. The sample was filtered through a 0.45 μm microfilter that was previously washed with 0.1% TFA solution before use and then directly loaded onto a HPLC C-18 column (Dialpack, Waters). Fractions were collected every tenth of a minute from 10 min after injection until 38.8 min containing 0.1% saponin (Sigma-Aldrich). Stained cells were analyzed on 1640 medium plus 10% FBS and 10 mM HEPES buffer and seeded at 2 × 10^5 L929 or HEK293 cells were infected at 10 MOI with rVV's expressing the IAV- derived peptides as minigenes for 6 hr before UV irradiation. After washing, 10 million cells were injected i.p. into each B6 mouse. Six days later, splenic and peritoneal L929 cells from primed animals were suspended in RPMI 1640 medium plus 10% FBS and 10 mM HEPES buffer and seeded at 2 × 10^6 cells/well in U-bottom, 96-well plates. Peptides were added to wells to a final concentration of 0.5 μM. Cells were incubated initially for 1–2 hr at 37°C and then for 3 hr with brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO) at 10 μg/ml. Cells were then stained with CyChrome anti-CDB1 mAb on ice for 30 min, washed, and fixed with 1% paraformaldehyde in PBS at room temperature for 20 min, then further stained with FITC-conjugated anti-IFN-γ in PBS containing 0.1% saponin (Sigma-Aldrich). Stained cells were analyzed on a FACSCalibur (BD Biosciences, Sunnyvale, CA) with live-gate on the CD8+ cells. A total of 150,000 cells were normally acquired and analyzed with FlowJo software (TreeStar, Ashland, OR).

**SUPPLEMENTAL DATA**

Two figures are available at http://www.immunity.com/cgi/content/full/28/6/787/DG1/.

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