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HIV ENV protein inhibits MHC class I presentation of a cytomegalovirus protective epitope[1]

Running Title: Endogenous peptide competition for binding to MHC class I

Daniel López*, Yolanda Samino*, Ulrich H. Koszinowski†, and Margarita Del Val*[2]

*Centro Nacional de Biología Fundamental. Instituto de Salud Carlos III. Madrid. Spain.

†Max-von-Pettenkofer Institute. Ludwig-Maximilian University of Munich. F. R. Germany.

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Summary

Cytotoxic T lymphocytes, CTL, recognize peptides that derive from viral protein antigens by proteolytic processing and are presented by MHC class I molecules. Here we tested if coexpression of viral antigens in the same cell leads to competition between them. To this end, two L^d-restricted epitopes derived from HIV-1 ENV glycoprotein and cytomegalovirus (CMV) pp89 phosphoprotein were coexpressed. HIV ENV strain IIIB but not MN variant impaired recognition by specific CTL of CMV 9pp89. Susceptibility to inhibition after ENV coexpression was inversely related to the amount of antigenic 9pp89 peptide processed from different antigenic constructs. In line with it, competition decreased the yield of naturally processed antigenic 9pp89 peptide bound to MHC class I molecules in coinfected cells. Also, point-mutants of the presenting MHC class I molecule differed in their competition pattern. Collectively, the data imply that competition operates at the step of MHC-peptide complex assembly or stabilisation. We conclude that, although not the rule, in certain combinations there is interference between different antigens expressed in the same cell and presented by the same MHC class I allele. These studies have implications for vaccine development and for understanding immunodominance.

Introduction

Viruses encode a high number of potential epitopes that can be presented by MHC class I molecules. Analysis of viral epitopes that are recognized in the infected host usually shows that the response to one epitope is much higher than to other potential epitopes, which are either not or only marginally recognized. Thus, one usually finds immunodominant and cryptic epitopes. If by mutation the pathogen loses the immunodominant epitope(s), some previously cryptic epitopes may now become immunodominant (1,2). This indicates that it was not intrinsic properties of these previously cryptic epitopes that precluded their efficient presentation to T lymphocytes. Rather, factors external to the epitope itself should account for it. It has been hypothesized that the presence of a very strong epitope renders cryptic another one of regular strength (2,3) because of competition between them, be it at the level of the infected cell, the presenting cell, or the $CD8^+$ T lymphocyte.

We are interested in studying if the co-expression of several antigens in the same cell compromises the efficiency of presentation to $CD8^+$-specific T cells, particularly when they are restricted by the same MHC class I molecule. The endogenous pathway of viral antigen presentation to CTL comprises several steps, including proteolytic processing by proteasomes or other enzymes (4-7), peptide transport to the endoplasmic reticulum (ER)[3] by the transporters associated with antigen processing (TAP), assembly of the peptide/MHC complex, and migration to the cell membrane. It is possible that different antigens may compete with each other at any of these stages. This question was addressed in a mouse model by studying the interference between two antigens recognized by $CD8^+$ CTL, both presented by the murine MHC class I molecule L^d (8,9). One is derived from HIV-1 strain IIIB, the immunodominant determinant ^{318}RGPGRAFVTI^{327} of the envelope glycoprotein ENV gp160, that is also presented by various murine: D^d (10), H-2^q, H-2^u, H-2^p (11) and human class I molecules: HLA-A2 (12), -A11 (13), -A3 (14), and -B27 (15). The second epitope is derived from murine CMV, corresponds to the immunodominant

^{168}YPHFMPTNL^{176} nonamer of the nuclear phosphoprotein pp89, and can elicit $CD8^+$ T lymphocytes that protect against lethal murine CMV infection (16-18). This report shows that HIV ENV interfered with presentation of CMV 9pp89 to CTL, and that this occurred at the level of processed peptides bound to MHC class I molecules in the coinfected cells.

Materials and Methods

Mice

BALB/c mice (H-2^d haplotype) were bred in our colony.

Cell lines

All cell lines were cultured in Iscove's modified Dulbecco's medium supplemented with 10% foetal calf serum and 1% 2-mercaptoethanol. Generation of site-directed L^d mutants and their transfection into murine L fibroblasts were described elsewhere (19,20). Transfectants are named indicating the new amino acid substituting for the native amino acid at the indicated residue number. Accordingly, transfectant R144K/R145H was renamed as L/L^d144K145H, AB as L/L^d107W116Y, L95 as L/L^d95I, L116 as L/L^d116Y, and A as L/L^d107W116Y155H157K. T2 cells transfected with L^d have been described (21).

Synthetic peptides

Peptides were synthesized in an Applied Biosystems peptide synthesizer model 431A, purified, and found homogeneous by reversed-phase HPLC. The sequences are as follows: 9pp89: YPHFMPTNL, 10ENV: RGPGRAFVTI, 9ENV: GPGRAFVTI, 9ENV-MN: GPGRAF**Y**T**T**.

Recombinant vaccinia viruses

Generation of rVV-eN-A9pp89A and rVV-HBe viruses has been described previously (22). The former encodes the murine CMV 9pp89 immunodominant epitope biterminally flanked by five alanines and inserted into the amino terminus of the secretory core protein of hepatitis B virus (HBe). Recombinant rVV-eC-10env encodes the HIV-1 strain IIIB ENV 318-327 epitope at the carboxyl end of HBe (7). The rVV-sN-9pp89S recombinant was generated similarly (22), and the sequence at the N-terminal

insertion site is ...mdiGYPHFMPTNLSdpy..., where the lower-case letters indicate the native HBe carrier protein N-terminal sequence. As opposed to the other HBe-based chimeras, this one contains the influenza virus hemagglutinin signal in place of the native HBe signal sequence, and the 9pp89 epitope is not flanked by alanines. rVV-ENV-IIIB virus (vSC25) encodes ENV envelope glycoprotein from the strain IIIB of HIV-1 (23). rVV-ENV-MN encodes ENV from strain MN of HIV-1 (10), that contains the homologous epitope ^{318}**I**GPGRAF**Y**T**T**^{327} presented by D^d (10). rVV-encoded proteins relevant to this study contain their respective signal sequences for translocation into the ER. All foreign genes cloned into the rVV used in this study are under the control of the vaccinia early-late promoter 7.5k. rVV-ENV-IIIB and rVV-ENV-MN are based on vaccinia WR strain whereas the others present Copenhagen background.

T cell lines and cytolytic assays

Polyclonal pp89- or ENV-IIIB-monospecific CTL were generated by immunization of mice with murine CMV or with rVV-ENV-IIIB as described elsewhere (5,6) and used as effector cells in standard cytolytic assays after overnight infection of transfectants with rVV as described (9). Percentage specific inhibition was calculated as:

$$\% \text{ Specific Inhibition} = 100 - \frac{[(\text{Lysis rVV-Antigen + competing rVV}) - \text{Lysis rVV-HBe}] \times 100}{\text{Lysis (rVV-Antigen + rVV-HBe)} - \text{Lysis rVV-HBe}}$$

For this calculation, the values from those E:T ratios that gave reliable lysis values, that is, equal or superior to half maximal lysis, from at least two independent experiments, were used. Hoffmann-La Roche generously provided recombinant human interleukin 2 for the long-term propagation of antigen-specific CTL lines.

Western blot

Cell pellets corresponding to 4 x 10^6 infected L/L^d cells were loaded onto SDS-PAGE

gels, transferred to Immobilon-P membranes (Millipore), and developed either with a rabbit antiserum to HBc/HBe (7), or with mAb 96.13.48 specific for the HIV-1 strain IIIB ENV epitope, kindly provided by Dr. A. Toraño (Instituto de Salud Carlos III, Madrid, Spain) (manuscript in preparation). Standard ECL Plus (Amersham) procedures were followed.

Cold target inhibition assays

^{51}Cr labeled P815 cells were prepulsed for 30 min with 1x10^{-6}M 9pp89. Cold targets were unlabeled P815, pulsed with 1x10^{-6}M 9ENV or 1x10^{-6}M 9pp89. All target cells were then washed and mixed for 30 min before adding pp89-specific CTL in a standard 3-hr cytolytic assay. The cold:hot target ratio was 30:1.

Isolation of naturally processed peptides

Transfectants were infected in parallel with the different mixtures of rVV at the indicated moi, and 16 hr later naturally processed peptides were extracted from whole cells with trifluoroacetic acid and purified essentially as previously described (22). Instead of gel filtration, Macrosep 3K centrifugal concentrators were used for the isolation of low-molecular-weight peptides. Reversed-phase HPLC fractions were tested in triplicate with ^{51}Cr-labeled P815 cells and pp89-specific CTL for their content in antigenic peptides. Serial dilutions of positive fraction 31 of HPLC runs, which corresponds to 9pp89 immunodominant nonamer, were tested in cytolytic assays performed always in parallel.

MHC/peptide stability assays

T2/L^{d} cells were incubated overnight at 26ºC, washed, and incubated for 2 h at 37ºC in PBS containing 0.1% bovine serum albumin and 500 μM of the different peptides. A control without peptide was also included. After washing, the cells were resuspended in the same buffer (time point 0 h) and further incubated at 37ºC. Aliquots removed at

different time points were stained with mAb 30-5-7, which recognizes L^d bound to peptide (24), and FITC-labeled goat anti-mouse antibody, or with the second antibody alone. Analysis of fixed cells was performed in a FACScan (Becton Dickinson). Fluorescence index was calculated at each time point as the ratio of mean linear fluorescence of the sample to that of the control incubated without peptide.

Results

Interference with antigen presentation

Coinfection experiments were performed with recombinant vaccinia viruses (rVV) that express either HIV-1 ENV or CMV pp89 optimal 9mer, named 9pp89, in an optimal chimeric protein context (22). Coinfection of rVV-eN-A9pp89A together with an excess of the control virus rVV-HBe did not affect the recognition of infected L/L^d fibroblasts by CMV-specific CTL (Fig. 1a). Vaccinia codes for some unidentified antigens presented by L^d (9), which by their nature and amount apparently do not prevent 9pp89 antigen processing and presentation by L^d (18), nor that of many other antigens (25). These results show that this is also true even when vaccinia antigens are expressed in 5-fold excess. Coinfection experiments with rVV-ENV-IIIB, however, practically abolished recognition of 9pp89 (Fig. 1a), with an average inhibition of 73% $\pm$ 13% (n=10). Coinfection experiments in the macrophagic cell line J774 led to similar specific inhibition of 9pp89 presentation caused by coexpression of ENV (83% $\pm$ 6%, n=5), while the negative control WR did not cause any interference (3%). These data showed that, although not the rule, in certain combinations there is interference between different antigens expressed in the same cell and presented by the same MHC class I allele.

Competition experiments with exogenously added synthetic peptides showed that incubation with an excess of HIV ENV 318-327 epitope led to inhibition of recognition by CTL of 9pp89-peptide-loaded L/L^d fibroblasts, indicating that ENV peptides can prevent MHC-restricted presentation of exogenous 9pp89 (Fig. 2).

To investigate if the interference observed in infected cells was caused by the L^d-restricted epitope of ENV-IIIB, a rVV was constructed that expressed this 318-327 epitope in a different protein context, that of the secretory HBe protein. Fig. 1b shows that the rVV-eC-10env virus also interfered with 9pp89 antigen presentation, although less efficiently than ENV (35% $\pm$ 13%, n=10). In part, this may be due to a lower

efficacy of ENV epitope processing and presentation from the former construct, since lysis by ENV-epitope-specific CTL of L/L^d cells revealed a stronger recognition of full-length ENV than of the epitope construct (data not shown). Conversely, when the natural MN variant of ENV that differs at the epitope sequence was used as coinfecting virus, no inhibition of CMV epitope presentation was detected (Fig. 1c). Again, a control WR virus with a different wild type background did not interfere either. The MN variant, as well as the synthetic peptide mimicking its sequence in the epitope area, were not recognized by ENV-IIIB-specific CTL in L/L^d cells either (Fig. 3). Altogether, these results indicate that the L^d-restricted ENV epitope alone is able to partially interfere.

As a control, the effect of coinfection with the different viruses on the level of expression of the antigenic protein eN-A9pp89A was checked by Western blot. As shown in Fig. 4 (upper panel), although coinfection with a 5-fold excess of another rVV somehow decreased the level of eN-A9pp89A protein, this happened to a similar degree with the interfering ENV-IIIB and with wild type WR. In addition, although coinfection with rVV-ENV-MN strongly affected eN-A9pp89A protein expression (lane 4), this was not sufficient to affect specific recognition of infected cells by CTL (Fig. 1c). A similar reduction in total protein level after coinfection was detected when protein expression was analyzed with an ENV-IIIB-specific mAb (Fig. 4, lower panel). Thus, in conclusion, ENV interfered with CMV 9pp89 antigen presentation and this could not be explained by a selective reduction in intracellular levels of the antigenic protein.

Lack of antagonism between CMV 9pp89 and HIV 9ENV synthetic peptides

The findings described above are compatible with two situations. The two full-length antigens or peptides derived from them may compete intracellularly in the processing and presentation pathway. Alternatively, peptides derived from both epitopes may bind separately to MHC, and ENV complexes may antagonize (26) pp89-specific CTL at the cell surface. The latter possibility was simulated with synthetic peptides. Uninfected cells loaded with 9ENV were used as cold inhibitors of ^{51}Cr-

labeled 9pp89-loaded target cells, and found not to block CTL recognition of 9pp89 (Fig. 5). As a positive control, when both cold and labeled cells were pre-loaded with 9pp89, inhibition of lysis was observed. Altogether, these results indicated absence of antagonism between both epitopes, suggesting that interference occurred within the infected cell.

Competition quantitatively affects the yield of naturally processed peptides

In order to confirm that the interference between both antigens occurred intracellularly, the amount of naturally processed peptides extracted from infected cells was assessed. Peptides that coelute in fraction 31 with the synthetic 9pp89 and that constitute the major antigenic activity of the CMV protein in reversed-phase HPLC runs were analyzed. Serial dilution of this material allowed quantitation of 9pp89 obtained from coinfection experiments with the control rVV-HBe or with the rVV that expressed the ENV epitope (rVV-eC-10env) (Fig. 6). The data showed 3-to-10-fold less rVV-eN-A9pp89A-derived 9pp89 obtained from cells coinfected with ENV rVV than with control rVV-HBe. Such a decrease in the yield of endogenously processed 9pp89 peptide was previously found to severely impair induction of protection immunity to murine CMV (22). No antigenicity was recovered from infected cells lacking L^d (data not shown). Thus, inefficient antigen presentation in ENV-epitope-expressing cells correlated with a lower amount of processed 9pp89 peptide bound to L^d. Collectively, these results suggested that antigen competition took place intracellularly before or at the step of peptide binding to L^d.

The degree of inhibition is inversely related to the amount of antigenic peptide

Because intracellular competition for antigen presentation correlated with lower amounts of naturally processed 9pp89 peptide, constructs that yield less processed 9pp89 should be easier to compete with.

rVV-eN-A9pp89A and rVV-sN-9pp89S viruses express the immunodominant

CMV epitope with different local flanking regions. Quantitation of naturally processed peptides from both chimeric proteins yielded 5-10 fold less 9pp89 peptide from rVV-sN-9pp89S (Luderer, E., Del Val, M., and Koszinowski, U. H., unpublished data), which correlated with target cell lysis. Fig. 7 shows coinfection followed by cytolysis using rVV-eC-10env as competing virus. The specific inhibition by rVV-eC-10env of rVV-sN-9pp89S (78% $\pm$ 14%) (n=3) was more prominent than that of rVV-eN-A9pp89A (35% $\pm$ 5%) at the same multiplicity of infection (moi) ratios (1:5 antigen:competitor). Thus, inhibition is stronger under conditions where less antigenic peptide is generated.

The reverse situation was also explored, namely, increasing the amount of naturally processed 9pp89 peptide by raising the moi of the antigen rVV-sN-9pp89S. When a 3-fold higher moi of 9 of rVV-sN-9pp89S was used, but the same antigen:competitor ratio was maintained, specific inhibition decreased from 78% to 43% $\pm$ 5% (n=4) (Fig. 7). This revealed that increasing amounts of antigenic peptide gradually reverse competition. These results also exclude a non-specific interference by vaccinia virus properties and support the antigen-specific nature of the interference.

Point mutations in the MHC class I molecule affect the competition pattern

If peptides would compete for binding to MHC molecules, then MHC mutants that affect the peptide groove and that selectively modulate binding of the antigenic peptide (20) or of the competitor should differ in their capacity to resist competition. Coinfection and cytolysis assays were performed using L cells transfected with different MHC class I molecule L^d mutants. Six transfectants including L/L^d from a panel of 11 MHC class I mutant cells (19,20) were able to present rVV-eN-A9pp89A to CTL and were tested for competition (Fig. 8). The results show that rVV-ENV-IIIB efficiently inhibited 9pp89 presentation in all cases, whereas inhibition by rVV-eC-10env was only partial, as described above for L/L^d. Transfectants L/L^d107W116Y155H157K, L/L^d95I, and L/L^d144K145H (the last two not shown) exhibited a similar reaction pattern as L^d-expressing target cells. In these transfectants the degree of inhibition by rVV-ENV was

on average 2-fold higher than the inhibition caused by rVV-eC-10env. In contrast, the two transfectants L/L^d116Y and L/L^d107W116Y differed from the previous pattern. Both displayed a significantly lower percentage of specific inhibition with rVV-eC-10env virus as compared to rVV-ENV, by a factor around 4 (Fig. 8). These data show that mutation of MHC class I molecules alters the competition hierarchy in cells that otherwise share the whole antigen processing and presentation machinery. Hence, interference appears to be the result of competition between different peptides for binding to L^d or mutants thereof. Therefore, competition probably operates at the step of MHC-peptide complex assembly or stabilisation.

To assess the relative stability of the complexes of the MHC molecule with the different peptides, TAP-deficient T2 cells transfected with L^d were used. Fig. 9 shows that the stability of the surface complexes with the 9pp89 peptide or with the strain IIIB 10ENV peptide was similar. Peptide 9ENV produced slightly less stable complexes. The homologous 9mer from the MN strain, for which no evidence of presentation by L^d has been reported, nevertheless bound to L^d, albeit with less stability. These results are compatible with the hypothesis that inhibition of 9pp89 peptide presentation is directly caused by the presence of processed ENV peptides at sites of peptide/MHC assembly.

Discussion

In this report we address the question of interference between simultaneously expressed antigenic epitopes. We demonstrate that coexpression of two epitopes, one derived from HIV-1 ENV IIIB, but not from MN, and the CMV 9pp89 epitope, decreases the presentation by MHC class I molecules of CMV 9pp89 to specific $CD8^+$ CTL. This is probably caused by intracellular competition of processed antigens for binding to MHC class I molecules. To our knowledge, this is the first report that describes such a selective competition for peptide presentation caused by HIV-1 ENV protein.

The interference by the full-length ENV protein is higher than by the L^d-restricted 318-327 epitope construct. Thus, besides the action of the epitope itself, a contribution of other unknown L^d-restricted epitopes or other properties of the ENV protein to inhibition appear likely. Interestingly, TAP-independent presentation of several HLA-restricted ENV epitopes has been described (27,28), which can act simultaneously with the TAP-dependent classical presentation pathway. In our murine system, presentation of the 318-327 epitope from full-length ENV is TAP-dependent (6), while it can follow both a TAP-dependent and a TAP-independent pathway from the chimeric protein (6,7). Thus, additional ENV-derived peptides processed also in the secretory pathway may constitute an extra source of peptides that may compete with 9pp89 in our experiments. Notably, preferential and specific proteolytic cleavage of ENV protein occurs between residues 315 and 316, adjacent to the L^d-restricted epitope, as detected in several cell lines (29,30) including the L cells used in our study (31). The long-time residence of ENV in the ER (28) would favor this cleavage. This may also explain the higher efficiency of recognition of the full-length ENV by ENV-epitope-specific CTL. The described O-glycosylation of ENV at this epitope sequence (32) may also contribute to the diversity of peptides with potential for competition with 9pp89. A third source of competing ENV-related peptides could be the extracellular space or the cell membrane (33). Thus, the efficient processing of ENV at several relevant subcellular sites may make it a good source of competing peptides of sufficient

affinity for L^d. Differences in the epitope itself, as shown here for strain MN, or in these other properties, might contribute to the differential pathogenicity of HIV strains.

The experiments with L^d mutants showed different relative competition strength of the two ENV-expressing competing constructs in transfectants that possess identical endogenous processing pathway and only differ in point-mutated MHC class I molecules. In addition, quantitation of naturally processed 9pp89 peptide in cells expressing the competitor showed lower amounts of antigenic peptide. Also, the amount of processed peptide that was produced under different experimental conditions correlated inversely with the susceptibility to competition by ENV. This set of data therefore suggest that competition is not due to preferential cleavage by the proteasome or to differential transport by TAP of any 9pp89-related precursor peptide (34). Rather, we place the step of competition at a level where MHC class I molecules are involved, particularly at the stage of MHC/peptide complex assembly and stability. Interference would thus be explained by competition between different peptides for binding to L^d. Complex formation occurs in the ER (35), which is where competition may primarily occur. Peptide exchange at a later step in the secretory pathway (36), associated with further points of ENV peptide generation, as discussed above, may contribute as well.

Competition should occur under conditions of limited resources. For example, only strongly binding peptides should complex with limited MHC class I molecules, whereas under excess amounts of MHC also weaker peptides would have the chance to bind. It has been estimated that TAP translocates 20,000 peptides every minute per cell into the ER, whereas only about 100 peptide-receptive MHC class I molecules are synthesized during this time (37). In such a situation of MHC shortage, it is feasible that a potentially higher production in a relevant site of efficiently binding ENV peptides would lead to a disadvantage for CMV epitope binding.

Deficient endogenous presentation of an antigen may arise from inefficiencies in any of the sequential processes of degradation, TAP-dependent transport and MHC

class I binding. In this study we showed that also the presentation of an immunodominant epitope is conditioned by the presence of another dominant epitope. Two studies on CTL responses to HIV (2) and lymphocytic choriomeningitis virus (1), showing that weak epitopes are suppressed in the presence of stronger epitopes, although these weak epitopes are able to generate a competent response if expressed in isolation, could be interpreted accordingly. Also, the reverse situation of what is reported here has been described, namely, competition between MHC class I alleles for presentation of overlapping viral epitopes, which resulted in suboptimal loading of one of the alleles *in vitro* (38). Altogether, competition for the same MHC class I molecule appears to represent a mechanism that can modulate presentation, while it may not operate for MHC class II (39).

These data highlight potential limitations in the design of recombinant vaccines expressing cocktails of immunodominant epitopes from HIV ENV and other pathogens. In multi-epitope vaccine studies not involving ENV, observations compatible with partial interference have not been studied further (40,41). When the ENV epitope was included, a substantial role of the relative location of two epitopes in the efficiency of class I presentation both *in vitro* and *in vivo* was seen (41). Whether or not antigen competition by ENV or other antigens is a frequent event cannot be decided yet, although different HIV ENV strains already differ in these properties. It is also not clear whether this would apply to all MHC molecules, since the results reported here also stress the importance of MHC sequence differences for the outcome of competition. From what is known about immunodominance of epitopes within a single pathogen (2,3,42), antigen competition might not be a rare event. The real situation may lie somewhere between strong competition and complete liberty of presentation. Previously, only intrinsic features of the epitope (MHC affinity, peptide liberation) and its necessary partners (T cell repertoire, antigen presenting cells) have been implicated as influencing immunodominance. Now we describe in detail the mechanism that underlies the further qualitative level that contributes to immunodominance, which is

the presence and properties of fellow epitopes. Thus, we show a way in which circumstances external to the epitope itself can preclude its presentation.

Moreover, coinfection of the same cells by HIV and CMV also occurs *in vitro* (43) as well as naturally, interestingly involving antigen presenting cells, particularly in the central nervous system (44,45). In patients with AIDS, CMV causes severe retinitis, is associated with neurological damage, and represents a recurrent primary pulmonary pathogen (46). If ENV would interfere with antigen presentation of human CMV or of other pathogens in AIDS patients as it does with mouse CMV, ~~Thus,~~ our finding may have relevance in CTL responses in early stages of asymptomatic HIV-infected individuals if they are infected with CMV and other pathogens.

In summary, in addition to the described properties of ENV to interact with the CD4 molecule on helper T lymphocytes (47) and to induce increased susceptibility to apoptosis in T cells (48), the phenomenon described here would contribute to impaired viral antigen presentation to CTL and to a strain-specific HIV-associated progressive loss of immune competence.

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References

1. Gallimore, A., J. Hombach, T. Dumrese, H. G. Rammensee, R. M. Zinkernagel, and H. Hengartner. 1998. A protective cytotoxic T cell response to a subdominant epitope is influenced by the stability of the MHC class I/peptide complex and the overall spectrum of viral peptides generated within infected cells. *Eur. J. Immunol. 28*:3301.
2. Borrow, P., H. Lewicki, X. P. Wei, M. S. Horwitz, N. Peffer, H. Meyers, Nelson, J. A., J. E. Gairin, B. H. Hahn, M. B. A. Oldstone, and G. M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med. 3*:205.
3. Nowak, M. A., R. M. May, R. E. Phillips, S. Rowlandjones, D. G. Lalloo, S. McAdam, P. Klenerman, B. Koppe, K. Sigmund, C. R. M. Bangham, and A. J. McMichael. 1995. Antigenic oscillations and shifting immunodominance in HIV- 1 infections. *Nature 375*:606.
4. Gil-Torregrosa, B. C., Castaño A. R., and M. Del Val. 1998. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the trans-Golgi network protease furin. *J. Exp. Med. 188*:1105.
5. Lopez, D. and M. Del Val. 1997. Selective involvement of proteasomes and cysteine proteases in MHC class I antigen presentation. *J. Immunol. 159*:5769.
6. Lopez, D., B. C. Gil-Torregrosa, C. Bergmann, and M. Del Val. 2000. Sequential cleavage by metallopeptidases and proteasomes is involved in processing HIV-1 ENV epitope for endogenous MHC class I antigen presentation. *J. Immunol. 164*:5070.
7. Gil-Torregrosa, B. C., A. R. Castaño, D. Lopez, and M. Del Val. 2000. Generation of MHC class I peptide antigens by protein processing in the secretory route by furin. *Traffic 1*:641.

8. Bergmann, C. C., L. Tong, R. V. Cua, J. L. Sensintaffar, and S. A. Stohlman. 1994. Cytotoxic T cell repertoire selection. A single amino acid determines alternative class I restriction. *J. Immunol. 152*:5603.

9. Del Val, M., H. Volkmer, J. B. Rothbard, S. Jonjic, M. Messerle, J. Schickedanz, M. J. Reddehase, and U. H. Koszinowski. 1988. Molecular basis of cytolytic T-lymphocyte recognition of the murine cytomegalovirus immediate-early protein pp89. *J. Virol. 62*:3965.

10. Takahashi, H., S. Merli, S. D. Putney, R. Houghten, B. Moss, R. N. Germain, and J. A. Berzofsky. 1989. A single amino acid interchange yields reciprocal CTL specificities for HIV-1 gp160. *Science 246*:118.

11. Shirai, M., C. D. Pendleton, and J. A. Berzofsky. 1992. Broad recognition of cytotoxic T cell epitopes from the HIV-1 envelope protein with multiple class I histocompatibility molecules. *J. Immunol. 148*:1657.

12. Dadaglio, G., A. Leroux, P. Langlade-Demoyen, E. M. Bahraoui, F. Traincard, R. Fisher, and F. Plata. 1991. Epitope recognition of conserved HIV envelope sequences by human cytotoxic T lymphocytes. *J. Immunol. 147*:2302.

13. Achour, A., S. Lemhammedi, O. Picard, J. P. M'Bika, J. F. Zagury, Z. Moukrim, A. Willer, F. Beix, A. Burny, and D. Zagury. 1994. Cytotoxic T lymphocytes specific for HIV-1 gp160 antigen and synthetic P18IIIB peptide in an HLA-A11-immunized individual. *AIDS Res. Hum. Retroviruses 10*:19.

14. Achour, A., O. Picard, J. P. Mbika, A. Willer, R. Snart, B. Bizzini, C. Carelli, A. Burny, and D. Zagury. 1993. Envelope protein and p18(IIIB) peptide recognized by cytotoxic T lymphocytes from humans immunized with human immunodeficiency virus envelope. *Vaccine 11*:609.

15. Jardetzky, T. S., W. S. Lane, R. A. Robinson, D. R. Madden, and D. C. Wiley. 1991. Identification of self peptides bound to purified HLA-B27. *Nature 353*:326.

16. Del Val, M., K. Munch, M. J. Reddehase, and U. H. Koszinowski. 1989. Presentation of CMV immediate-early antigen to cytolytic T lymphocytes is selectively prevented by viral genes expressed in the early phase. *Cell 58*:305.

17. Del Val, M., H. J. Schlicht, H. Volkmer, M. Messerle, M. J. Reddehase, and U. H. Koszinowski. 1991. Protection against lethal cytomegalovirus infection by a recombinant vaccine containing a single nonameric T-cell epitope. *J. Virol. 65*:3641.

18. Jonjic, S., M. Del Val, G.M. Keil, M. J. Reddehase, and U. H. Koszinowski. 1988. A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. *J. Virol. 62*:1653.

19. Solheim, J. C., B. M. Carreno, J. D. Smith, J. Gorka, N. B. Myers, Z. Wen, J. M. Martinko, D. R. Lee, and T. H. Hansen. 1993. Binding of peptides lacking consensus anchor residue alters H-2L^{d} serologic recognition. *J. Immunol. 151*:5387.

20. Hengel, H., K. Burke, D. Kyburz, R. M. Zinkernagel, and U. H. Koszinowski. 1995. Peptides control the gain and loss of allele specificity by mutated MHC class I molecules. *J. Immunol. 154*:4557.

21. Crumpacker, D. B., J. Alexander, P. Cresswell, and V. H. Engelhard. 1992. Role of endogenous peptides in murine allogenic cytotoxic T cell responses assessed using transfectants of the antigen-processing mutant 174xCEM.T2. *J. Immunol. 148*:3004.

22. Del Val, M., H. J. Schlicht, T. Ruppert, M. J. Reddehase, and U. H. Koszinowski. 1991. Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. *Cell 66*:1145.

23. Chakrabarti, S., M. Robert-Guroff, F. Wong-Staal, R. C. Gallo, and B. Moss. 1986. Expression of the HTLV-III envelope gene by a recombinant vaccinia virus. *Nature 320*:535.

24. Ozato, K., T. H. Hansen, and D. H. Sachs. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2L^{d} antigen, the products of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol. 125*:2473.

25. Bennink, J. R. and J. W. Yewdell. 1990. Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function. *Curr. Top. Microbiol. Immunol. 163*:153.

26. Jameson, S. C., F. R. Carbone, and M. J. Bevan. 1993. Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells. *J. Exp. Med. 177*:1541.

27. Hammond, S. A., R. C. Bollinger, T. W. Tobery, and R. F. Silliciano. 1993. Transporter-independent processing of HIV-1 envelope protein for recognition by CD8^{+} T cells. *Nature 364*:158.

28. Hammond, S. A., R. P. Johnson, S. A. Kalams, B. D. Walker, M. Takiguchi, J. T. Safrit, R. A. Koup, and R. F. Siliciano. 1995. An epitope-selective, transporter associated with antigen presentation (TAP)-1/2-independent pathway and a more general TAP-1/2-dependent antigen-processing pathway allow recognition of the HIV-1 envelope glycoprotein by CD8^{+} CTL. *J. Immunol. 154*:6140.

29. Rodriguez, D., J. R. Rodriguez, J. F. Rodriguez, D. Trauber, and M. Esteban. 1989. Highly attenuated vaccinia virus mutants for the generation of safe recombinant viruses. *Proc. Natl. Acad. Sci. U.S.A. 86*:1287.

30. Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F.M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg. 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature 345*:622.

31. Rodriguez, D., J. R. Rodriguez, and M. Esteban. 1995. Enhanced proteolytic processing of the human immunodeficiency virus type 1 envelope protein in murine Ltk^{-} cells. *AIDS Res. Hum. Retroviruses 11*:81.

32. Hansen, J.E., H. Clausen, S.L. Hu, J.O. Nielsen, and S. Olofsson. 1992. An O-linked carbohydrate neutralization epitope of HIV-1 gp120 is expressed by HIV-1 ENV gene recombinant vaccinia virus. *Arch.Virol. 126*:11.

33. Kozlowski, S., M. Corr, M. Shirai, L. F. Boyd, C. D. Pendleton, J. A. Berzofsky, and D. H. Margulies. 1993. Multiple pathways are involved in the extracellular processing of MHC class-I-restricted peptides. *J. Immunol. 151*:4033.

34. Knuehl, C., P. Spee, T. Ruppert, U. Kuckelkorn, P. Henklein, J. Neefjes, and P.M. Kloetzel. 2001.The murine cytomegalovirus pp89 immunodominant H-2L(d) epitope is generated and translocated into the Endoplasmic Reticulum as an 11-mer precursor peptide. *J.Immunol. 167*:1515.

35. Del Val, M., H. Hengel, H. Hacker, U. Hartlaub, T. Ruppert, P. Lucin, and U. H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J. Exp. Med. 176*:729.

36. Day, P. M., F. Esquivel, J. Lukszo, J. R. Bennink, and J. W. Yewdell. 1995. Effect of TAP on the generation and intracellular trafficking of peptide-receptive major histocompatibility complex class I molecules. *Immunity 2*:137.

37. Momburg, F., J. J. Neefjes, and G. J. Hammerling. 1994. Peptide selection by MHC-encoded TAP transporters. *Curr. Opin. Immunol. 6*:32.

38. Tussey, L. G., S. Rowlandjones, T. S. Zheng, M. J. Androlewicz, P. Cresswell, J. A. Frelinger, and A. J. McMichael. 1995. Different MHC class I alleles compete for presentation of overlapping viral epitopes. *Immunity 3*:65.

39. Lo-Man, R., J. P. Langeveld, P. Martineau, M. Hofnung, R. H. Meloen, and C. Leclerc. 1998. Immunodominance does not result from peptide competition for MHC class II presentation. *J. Immunol. 160*:1759.

40. Thomson, S. A., S. L. Elliott, M. A. Sherritt, K. W. Sproat, B. E. H. Coupar, Scalzo, A. A., C. A. Forbes, A. M. Ladhams, X. Y. Mo, R. A. Tripp, P. C. Doherty,

D. J. Moss, and A. Suhrbier. 1996. Recombinant polyepitope vaccines for the delivery of multiple CD8 cytotoxic T cell epitopes. *J. Immunol. 157*:822.

41. Bergmann, C. C., Q. Yao, C. K. Ho, and S. L. Buckwold. 1996. Flanking residues alter antigenicity and immunogenicity of multi-unit CTL epitopes. *J. Immunol. 157*:3242.
42. Deng, Y., J. W. Yewdell, L. C. Eisenlohr, and J. R. Bennink. 1997. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. *J. Immunol. 158*:1507.
43. McKeating, J. A., P. D. Griffiths, and R. A. Weiss. 1990. HIV susceptibility conferred to human fibroblasts by cytomegalovirus-induced Fc receptor. *Nature 343*:659.
44. Webster, A. 1991. Cytomegalovirus as a possible cofactor in HIV disease progression. *J. Acquir. Immune. Defic. Syndr. 4 Suppl 1*:47.
45. Nelson, J. A., C. Reynolds-Kohler, M. B. A. Oldstone, and C. A. Wiley. 1988. HIV and HCMV coinfect brain cells in patients with AIDS. *Virology 165*:286.
46. Waxman, A. B., S. J. Goldie, H. Brettsmith, and R. A. Matthay. 1997. Cytomegalovirus as a primary pulmonary pathogen in AIDS. *Chest 111*:128.
47. Landau, N. R., M. Warton, and D. R. Littman. 1988. The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. *Nature 334*:159.
48. Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K. M. Debatin, and P. H. Krammer. 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature 375*:497.

Footnotes

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[2]Address correspondence to: Margarita Del Val, Centro Nacional de Biología Fundamental. Instituto de Salud Carlos III. Ctra. Pozuelo, km 2. E-28220 Majadahonda (Madrid). Spain. E-mail: mdval@isciii.es. Phone: 34 915 097 943. Fax: 34 915 097 919.

[3]Abbreviations: CMV: cytomegalovirus; 9pp89: murine CMV pp89 CTL epitope, of sequence YPHFMPTNL; ER: Endoplasmic reticulum; rVV: recombinant vaccinia virus; HPLC: high-performance liquid chromatography; moi: multiplicity of infection; TAP: transporters associated with antigen processing.

Figure Legends

Figure 1.- Cytolysis assays with coinfecting recombinant vaccinia viruses.

Infection of L/L^d fibroblast target cells with rVV-eN-A9pp89A (named "Ag") at moi 3 and with the following competing viruses at moi 15: rVV-ENV-IIIB, rVV-eC-10env, rVV-ENV-MN, and rVV-HBe. L/L^d cells were coinfected with the corresponding viruses overnight and then used as target cells for recognition by pp89-specific CTL lines. The code used is: rVV-eN-A9pp89A (positive control, ♦), rVV-eN-A9pp89A + rVV-HBe (●), rVV-eN-A9pp89A + rVV-ENV-IIIB (◆), rVV-eN-A9pp89A + rVV-eC-10env (■), rVV-eN-A9pp89A + rVV-ENV-MN (⧫), rVV-eN-A9pp89A + rVV-WR (❍), and rVV-HBe (solid line) used as negative control. Consistent results were obtained over a period of several months using different stocks of all viruses. As a positive control, recognition by CTL of rVV-HBe-infected cells prepulsed with 10^{-6} M 9pp89 synthetic peptide gave lysis values of 60, 49, and 29% at E:T ratios of 20, 5, and 1:1, respectively.

Figure 2.- Competition by ENV synthetic peptides for 9pp89 exogenous presentation.

Cytolysis experiments were carried out using $1x10^{-10}$M 9pp89 and different 10ENV (black bars) or 9ENV (white bars) concentrations. Each mix of 9pp89 and an ENV peptide was preincubated with L/L^d target cells prior to addition of anti-pp89 CTL. A 9pp89 concentration of 10^{-10} M was chosen because it was the lowest one that still gave maximal CTL recognition in the absence of ENV peptides.

Figure 3.- Strain-specific recognition of ENV by CTL.

L/L^d cells were infected with rVV-ENV-IIIB (♦) or rVV-ENV-MN (●), or with the negative control rVV-HBe (solid line), at moi of 10. A standard cytolytic assay was performed using CTL generated by immunizing mice with rVV-ENV-IIIB.

Figure 4.- Western blot analysis of coinfected cells.

L/L^d cells were infected with rVV-eN-A9pp89A alone (lanes 1) or coinfected with rVV-ENV-IIIB (lanes 2), with rVV-WR (lanes 3) or with rVV-ENV-MN (lanes 4). Cells in lanes 5 were infected with rVV-ENV-IIIB alone. Reactivity with an HBc/HBe-specific serum to reveal eN-A9pp89A is shown above, while detection with an HIV strain IIIB ENV epitope-specific mAb is shown below.

Figure 5.- Cold target inhibition assays

Labeled (*) P815 target cells were preincubated with 9pp89 (◆). As unlabeled "cold" target cells, P815 were used that were preincubated with either 9ENV (●) or 9pp89 (■). P815 cells without peptide were used as negative control (♦). The cytolytic assay of the mixtures of labeled and cold targets was performed with pp89-specific CTL. Similar results as those shown for P815 cells were also obtained with L/L^d cells (data not shown).

Figure 6.- Reduced yield of naturally processed antigenic 9pp89 peptide produced in cells coexpressing ENV epitope.

L/L^d cells were infected overnight with rVV in the following combinations: rVV-eN-A9pp89A + rVV-HBe (♦), and rVV-eN-A9pp89A + rVV-eC-10env (●). The first virus in each combination ("Ag") was used at a moi of 3 and the competing virus was used at a moi of 15. Acid-soluble molecules were extracted and separated by reversed-phase HPLC. Fractions were tested with pp89-specific CTL for their content in antigenic peptides. Specific lysis of serial dilutions of positive fraction 31 of HPLC runs, which corresponds to 9pp89 immunodominant nonamer, is shown on the vertical axis as a measure of the amount of the relevant antigenic peptide. The calculated number of infected cells from which peptides were recovered is given on the horizontal axis. ENV-derived antigenic peaks elute before fraction 15.

Figure 7.- Inverse correlation between the relative amount of peptides derived from 9pp89 constructs and their susceptibility to competition by ENV epitope.

Coinfection experiments were performed as described in Fig. 1 with the indicated rVV. Moi used is indicated in brackets. Lysis of cells coinfected with control rVV-HBe and either with rVV-eN-A9pp89A or with rVV-sN-9pp89S at moi 3 or 9, which were used to calculate specific inhibition, were 40%, 21%, and 35%, respectively. Lysis of rVV-HBe-infected cells was 2%.

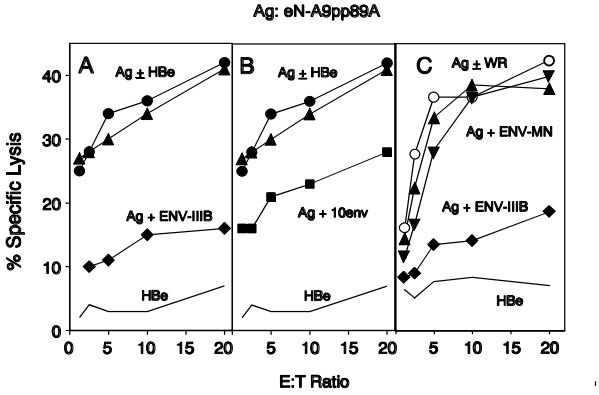
Figure 8.- Competition in different MHC class I transfectants.

L cells transfected with L^d mutated in the indicated positions and amino acids were coinfected with rVV-eN-A9pp89A at moi 3 and the rVV indicated on the left as competing viruses at moi 15. They were then used as target cells. Lysis of cells coinfected with rVV-eN-A9pp89A and control rVV-HBe, which were used to calculate specific inhibition, were 24%, 30%, 25%, 38%, 25%, and 35%, for transfectants L/L^d107W116Y, L/L^d116Y, L/L^d107W116Y155H157K, L/L^d95I, L/L^d144K145H, and L/L^d, respectively. The column at the right indicates the ratio of the inhibition caused by ENV to that caused by eC-10env. The factor for the transfectants not shown was 2.8, 2.4, and 2.1, for L/L^d95I, L/L^d144K145H, and L/L^d, respectively.

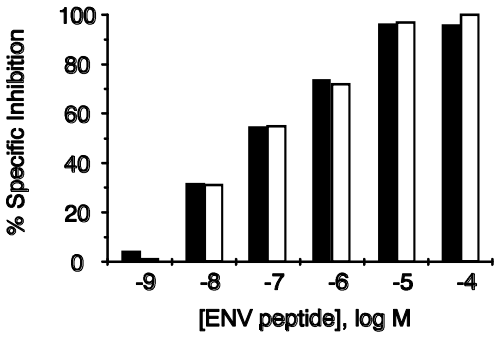
Figure 9.- Stability of MHC/peptide complexes.

After loading of $T2/L^d$ cells with the indicated peptides, these were washed away and the stability of the surface MHC/peptide complexes was estimated by staining the cells at different time points with the peptide-dependent L^d-specific 30-5-7 mAb, followed by flow cytometry. Peptides: 9pp89 (♦), 10ENV from IIIB (◆), 9ENV from IIIB (●), 9ENV-MN (■). Cells incubated without peptide had mean fluorescence intensities close to background staining with second antibody alone. Results are the mean of three different experiments.

Figures



López et al. Figure 1



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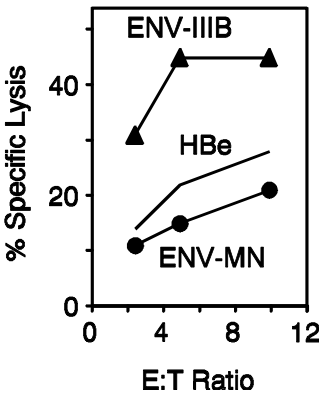

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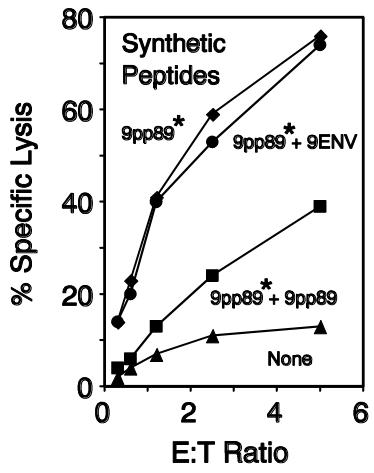

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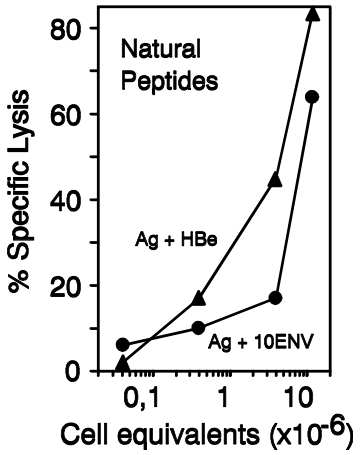

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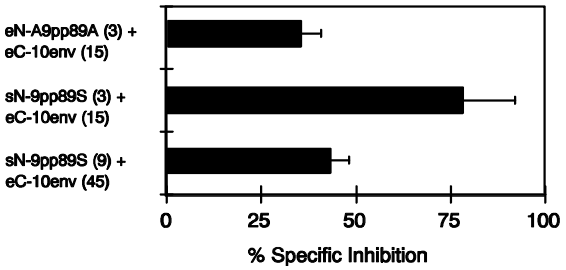

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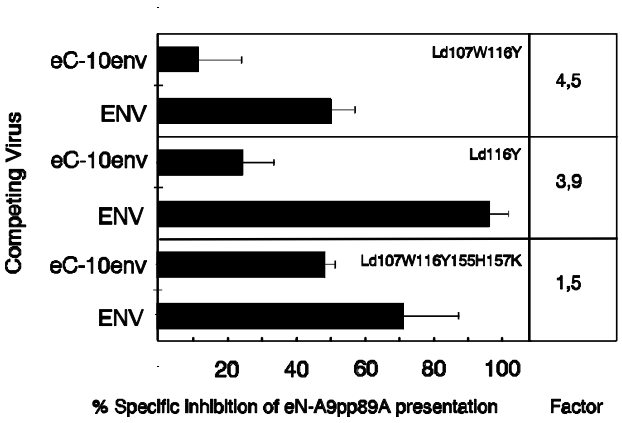

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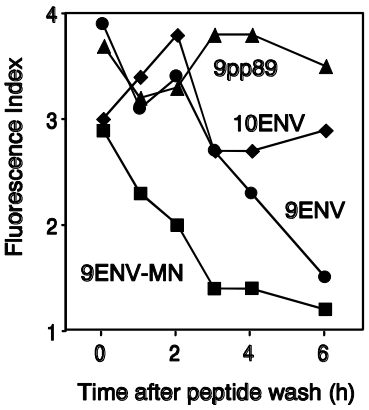

López et al. Figure 9