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IFN-γ Exposes a Cryptic Cytotoxic T Lymphocyte Epitope in HIV-1 Reverse Transcriptase[1]

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The proteasome, an essential component of the ATP-dependent proteolytic pathway in eukaryotic cells, is responsible for the degradation of most cellular proteins and is believed to be the main source of MHC class I-restricted antigenic peptides for presentation to CTL. Inhibition of the proteasome by lactacystin or various peptide aldehydes can result in defective Ag presentation, and the pivotal role of the proteasome in Ag processing has become generally accepted. However, recent reports have challenged this observation. Here we examine the processing requirements of two HLA A*0201-restricted epitopes from HIV-1 reverse transcriptase and find that they are produced by different degradation pathways. Presentation of the C-terminal ILKEPVHGV epitope is impaired in ME275 melanoma cells by treatment with lactacystin, and is independent of expression of the IFN-γ-inducible proteasome β subunits LMP2 and LMP7. In contrast, both lactacystin treatment and expression of LMP7 induce the presentation of the N-terminal VIYQYMDDL epitope. Consistent with these observations we show that up-regulation of LMP7 by IFN-γ enhances presentation of the VIYQYMDDL epitope. Hence interplay between constitutive and IFN-γ-inducible β-subunits of the proteasome can qualitatively influence Ag presentation. These observations may have relevance to the patterns of immunodominance during the natural course of viral infection. ***The Journal of Immunology*****, 1999, 162: 7075–7079.**

The proteasome, a nonlysosomal proteinase complex, plays a critical role in the degradation of intracellular proteins and is believed to be the main source of MHC class I-restricted antigenic peptides for CTL. Inhibition of the proteasome by lactacystin or various peptide aldehydes has been reported to result in defective Ag presentation (1–3), and the role of the proteasome in Ag processing has become generally accepted. The catalytic core of the proteasome, the 20S proteasome, has a cylindrical structure made up of 14 α and 14 β subunits. The β subunits bear the catalytic activity. Three of these β subunits, LMP2, LMP7, and MECL-1, are IFN-γ inducible and can replace the constitutive catalytic subunits delta, MB1, and Z, respectively (4, 5). Studies of mice deficient in either LMP2 or LMP7 indicate that these IFN-γ-inducible subunits may play a role in Ag processing. LMP2-deficient mice have fewer mature CD8 T cells and a diminished CTL response to influenza virus infection (6). Mice lacking LMP7 have diminished cell surface expression of MHC class I and present an H-Y H-2D^b epitope poorly (7). To date there are no data on mice lacking both LMP2 and LMP7, or mice deficient in MECL1. Recent evidence indicates that incorporation of LMP2 and MECL1 into proteasomes requires LMP7 favoring the assembly of homogeneous "immunoproteasomes" containing all three inducible subunits (8). Thus there are at least two types of proteasome within a cell; those which include the constitutively expressed catalytic subunits and immunoproteasomes incorporating the MHC-encoded subunits. These two proteosomes have different cleavage specificities (9–11).

HLA A*0201 is the dominant class I molecule in Caucasoid and other populations (12). Many HLA A*0201 HIV-1 infected patients mount a response to an immunodominant epitope encoded within the *gag* gene (residues 77–85; numbered with reference to HIV-LAI). This epitope, SLYNTVATL, is part of the p17 matrix protein. We have documented several naturally occurring mutations within this epitope (13). In a study of 22 HIV-1-infected individuals with HLA A*0201, we found that 15 made a CTL response to this epitope (14). Patients with HLA A*0201 who do not make a CTL response to SLYNTVATL often have epitope mutations and respond to the reverse transcriptase (RT)[4] epitope ILKEPVHGV (Pol residues 476-484) (14). The pattern of immunodominance of CTL epitopes in HIV-1 is not fully understood. Gag epitope outnumbers the RT epitope by over 30 times on the surface of stably infected Jurkat A2 cells (15). This may be caused by differences in the efficiency with which these peptides are produced. We also examined a further HLA A2-restricted epitope in HIV-1 RT of sequence VIYQYMDDL (Pol residues 346-354). The VIYQYMDDL RT epitope contains the core sequence YMDD, which constitutes the active site of RT (16). This sequence is invariant in HIV-1 (17, 18) and is conserved among RNA-dependent DNA polymerases from other human and animal retroviruses (16). Accordingly, this epitope is severely restricted in its ability to mutate and escape from CTL pressure.

Within HIV-1 RT, ILKEPVHGV appears to be the dominant HLA A*0201-restricted epitope (A. K. Sewell and D. A. Price,

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[4] Abbreviations used in this paper: RT, reverse transcriptase; RT-Vac, the vaccinia expressing HIV-1 RT; MOI, multiplicity of infection; Gag-Vac, HIV-1 Gag expressing vaccinia; .45 cells, LCL721.45 cells; .174 cells, LCL721.174 cells.

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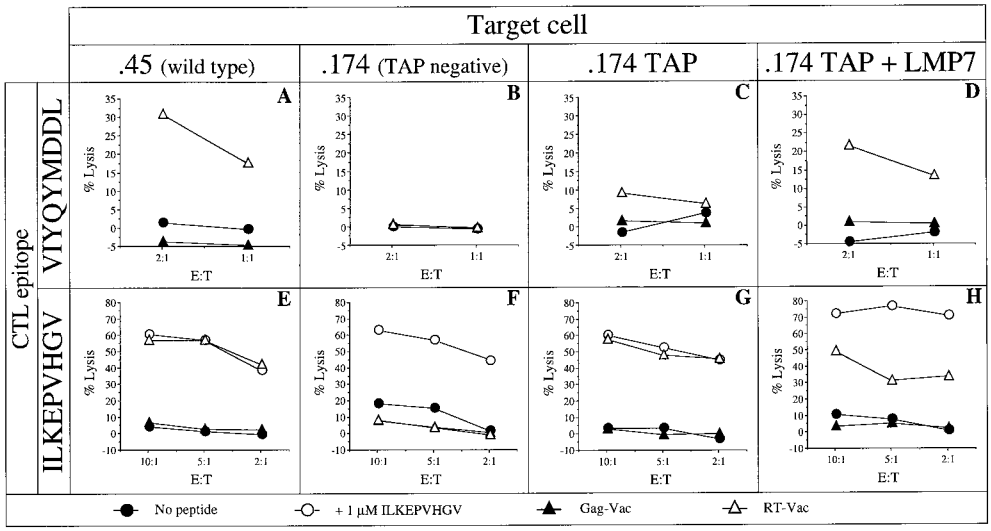

FIGURE 1. Presentation of VIYQYMDDL, but not ILKEPVHGV, from HIV-1 RT is dependent on LMP7 expression. Target cells (.45, .174, .174 TAP or .174 TAP + LMP7) were either uninfected or infected with recombinant vaccinia expressing HIV-1 RT or an irrelevant vaccinia (Gag-Vac). A total of 5×10^3 targets in triplicate in 96-well U-bottom plates were presented to CTL specific for the RT epitopes VIYQYMDDL and ILKEPVHGV (*A* to *D* and *E* to *H*, respectively) at the E:T ratios indicated. The percentage of lysis is shown for uninfected cells with (○) and without (●) the addition of 1 μM ILKEPVHGV peptide to the wells and for targets after overnight infection with a recombinant vaccinia expressing HIV-1 RT (△) or irrelevant vaccina (▲). Infection with the appropriate vaccinia sensitizes cells for lysis by all CTL in wild-type .45 cells, which have functional TAP, LMP2, and LMP7 genes (*A* and *E*). .174 TAP cells, which lack the LMP genes, fail to present the VIYQYMDDL epitope (*C*) at levels that elicit lysis by CTL. .174 TAP cells are unhindered in their ability to process and present the other HLA A2-restricted RT epitope ILKEPVHGV (*G*). Transfection of .174 TAP cells with LMP7 restores wild-type levels of target lysis VIYQYMDDL (*D*).

unpublished observation). Since the rules that govern the dominance of Ags are crucial to the understanding of immunity to pathogens, we were interested in the processing requirements of these two RT epitopes. While examining the role of the proteasome in their production, we found that these epitopes appear to be the result of different protein degradation pathways.

Materials and Methods

Cell culture

LCL721.45 cells (.45), a wild-type derivative of LCL721, and LCL721.174 cells (.174), which has only one copy of chromosome 6 containing a deletion in the class II region of the MHC locus and therefore lacks the TAP1 and 2, LMP2, and LMP7 genes, are described elsewhere (19). Transfection of .174 cells with TAP1 and 2 is described elsewhere (20). The HIV-1 Pol (ILKEPVHGV) CTL line from patient SC6 and the VIYQYMDDL clone have been described previously (21). HeLa cells transfected with HLA A2 were a kind gift of Dr. H. Stauss (Hammersmith Hospital, London, U.K.). 174 cells expressing the TAP1 and TAP2 genes were transfected with plasmid pCEP4Δ expressing the human LMP7b gene as described elsewhere.[5]

Vaccinia infection

Target cells were infected with vaccinia at a multiplicity of infection (MOI) of 5 for 90 min and, after washing, resuspended in R10 (RPMI 1640 with added glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and FCS (10% v/v)). Infected cells were grown overnight; shorter grow out times (<6 h) were not sufficient to produce significant lysis by CTL. The vaccinia expressing HIV-1 reverse transcriptase (RT-Vac; vCF21) is described elsewhere (22) and was obtained from the AIDS Reagent Program (Rockville, MD). An HIV-1 Gag expressing vaccinia (Gag-Vac) was used as a specificity control. This vaccinia has been described previously (23) and is available from the MRC AIDS Reagent Project (Potters Bar, U.K.).

[5] I. Correa, H. Teisserenc, L. Van Kaer, H. J. Fehling, V. Cerundolo, and J. Trowsdale. LMP7 is a key subunit in the assembly of the immunoproteasome and in the presentation of an immunodominant influenza virus epitope to cytotoxic T lymphocytes. *Submitted for publication.*

Cytolytic assays

CTL assays were performed as described previously (23). Details of individual experiments are given in the figure legends.

Lactacystin treatment of target cells

Cells (10^6) were resuspended in 50 μl of R10 medium containing 100 μM lactacystin for 1 h prior to addition of vaccinia in 50 μl at MOI 5. After 90-min infection, cells were washed and resuspended in 5 ml of R10 containing 1 μM lactacystin and grown overnight to allow expression of the RT gene.

Treatment with IFN-γ

IFN-γ (R&D Systems, Abingdon, U.K.) was added to culture medium at 200 U/ml for 48 h prior to subsequent vaccinia infections and lysis assays.

Western blots

A total of 7 μg of total protein from indicated cell lines was separated by SDS-PAGE and Western blotted for LMP7 and MB1 as described previously (4).

Results

The cell line LCL721.174 (.174) contains a large deletion in the MHC class II region (19) and lacks the TAP genes, and the LMP2 and 7 genes. These cells are unable to present either the ILKEPVHGV or VIYQYMDDL epitope from RT (Fig. 1, *B* and *F*). Addition of the TAP1 and 2 genes (.174 TAP) restores presentation of ILKEPVHGV, but presentation of VIYQYMDDL is not significantly enhanced (Fig. 1). Further transfection of .174 TAP cells with LMP7 restores presentation of the VIYQYMDDL epitope in HIV-1 RT to levels seen in wild-type cell .45 (Fig. 1). LMP7 can substitute the MB1 subunit in the proteasome and alter cleavage specificity (4, 5, 9–11). Consequently, constitutive expression of LMP7 in .174 cells may alter the cleavage pattern of HIV-1 RT so as to generate VIYQYMDDL. Alternatively, LMP7 could be directly involved in generation of the VIYQYMDDL epitope. Lactacystin treatment of .174 TAP cells transfected with

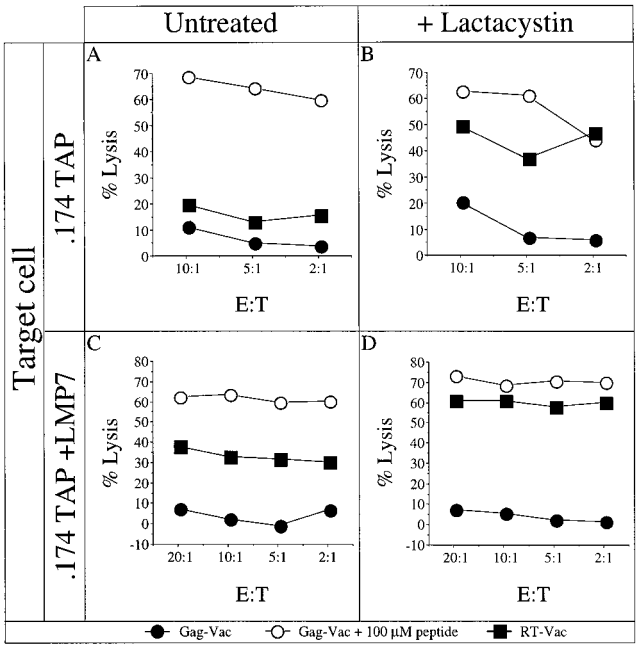

FIGURE 2. The effects of LMP7 expression and lactacystin treatment on presentation of VIYQYMDDL. As in Fig. 1 (except target cells), either .174 TAP (*A* and *B*) or .174 TAP transfected with LMP7 (*C* and *D*) were either left untreated (*A* and *C*) or pretreated with lactacystin (*B* and *D*) prior to infection with vaccinia as described in *Materials and Methods*. Target cells were incubated with CTL specific for the VIYQYMDDL epitope. Each point represents the mean of three replicates. Cells infected with Gag-Vac were used as a negative control, and positive control in the presence of 100 μM peptide. Treatment of .174 TAP cells with lactacystin, like expression of LMP7, restores their ability to process and present the VIYQYMDDL epitope (*B*). Paradoxically, treatment of .174 TAP + LMP7 cells with lactacystin fails to reverse the positive effects of LMP7 expression (*D*).

LMP7 did not reduce presentation of VIYQYMDDL (Fig. 2, *C* and *D*). While lactacystin may not block all of the activities of the proteasome, it is known to bind the active site of LMP7 and its constitutively expressed counterpart MB1 (1, 24). It is paradoxical that the catalytic potential of LMP7 is not responsible for generation of VIYQYMDDL from RT but that expression of this subunit can lead to effective presentation of this epitope. The ability of the normally IFN-γ-inducible LMP7 to displace the constitutively produced catalytic subunit MB1 (4) may provide an explanation. Schmidtke et al. (25) recently documented that substitution of the delta subunit of the constitutive proteasome by the LMP2 subunit enhanced presentation of a class I epitope.

We postulated that the constitutive proteasome, containing the MB1 subunit, may be responsible for destroying the VIYQYMDDL RT epitope. If this were true, then inhibition of the constitutive proteasome should remove its destructive effect on the generation of this epitope. Indeed, treatment of .174 TAP cells, which lack LMP7, with lactacystin before infection with a recombinant vaccinia expressing HIV-1 RT restored presentation of VIYQYMDDL (Fig. 2). Consequently, inhibition of the constitutive proteasome, either by treatment with lactacystin or by constitutive expression of LMP7, thereby substituting MB1, leads to efficient generation of the VIYQYMDDL RT epitope. Thus it is possible that digestion of HIV-1 RT by the constitutive proteasome destroys the VIYQYMDDL epitope. The increase in recognition observed when .174 TAP + LMP7 cells are treated with lactacystin (Fig. 2, *C* and *D*) could be due to lactacystin inhibition of the destructive effects of proteasomes containing residual MB1 subunit in these cells.

To further address whether the constitutive proteasome destroys VIYQYMDDL, we examined the effects of lactacystin treatment on the presentation of both RT epitopes in melanoma and HeLa cell lines which, unlike .45 cells, constitutively express MB1 but little LMP7 by Western blot (Fig. 3*A* and 4*C*). The melanoma cell line ME275 was infected with recombinant vaccinia virus expressing HIV-1 RT, and presentation of both the VIYQYMDDL and ILKEPVHGV epitopes was examined simultaneously. Figure 3 shows that only the ILKEPVHGV epitope is efficiently presented in untreated cells. Treatment of the melanoma cell line with lactacystin rescues the generation of VIYQYMDDL while presentation of ILKEPVHGV is impaired. This result was repeated eight times; in HeLa cells and two other HLA *0201 melanoma cell lines (data not shown). Treatment of target cells with 350 μM Z-LLnL-CHO, which also inhibits the proteasome (2), had the

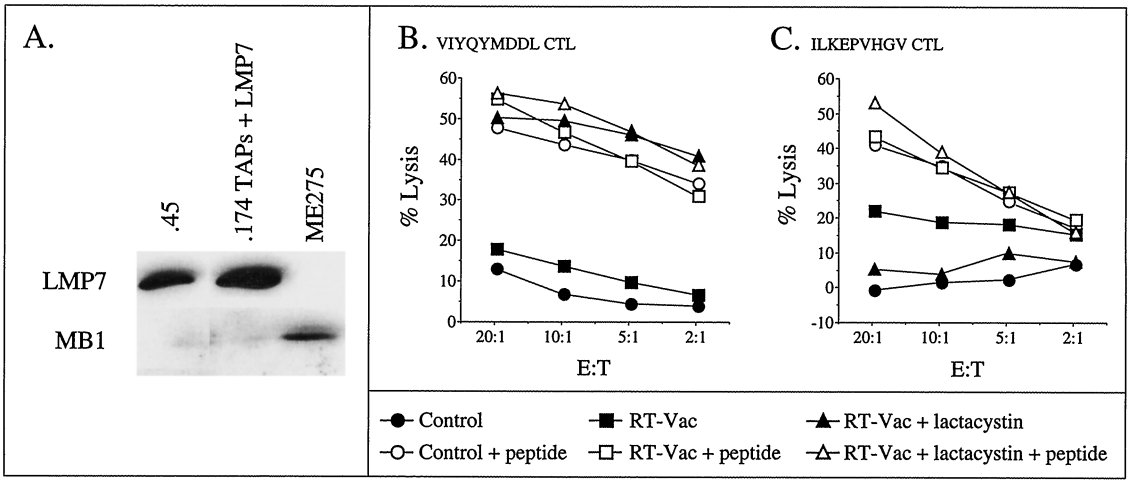

FIGURE 3. Two HLA A*0201-restricted epitopes in HIV-1 RT are produced by different degradation pathways. *A*, The melanoma cell line ME275 expresses MB1 but little of the IFN-γ-inducible subunit LMP7 by Western blot. *B* and *C*, As for Fig. 1 except using the melanoma cell line ME275 as target cells. Targets were presented to CTL specific for both HIV-1 RT epitopes at indicated E:T ratios. All data points represent the mean of three replicates. The N-terminal VIYQYMDDL and the C-terminal ILKEPVHGV epitopes are produced by two distinct degradation pathways. The N-terminal epitope is not presented efficiently in ME275 cells; lactacystin treatment enhances lysis (*B*). The C-terminal epitope is presented in the absence of lactacystin treatment. Treatment with lactacystin causes a failure to process and present this Ag in quantities sufficient to activate CTL (*C*).

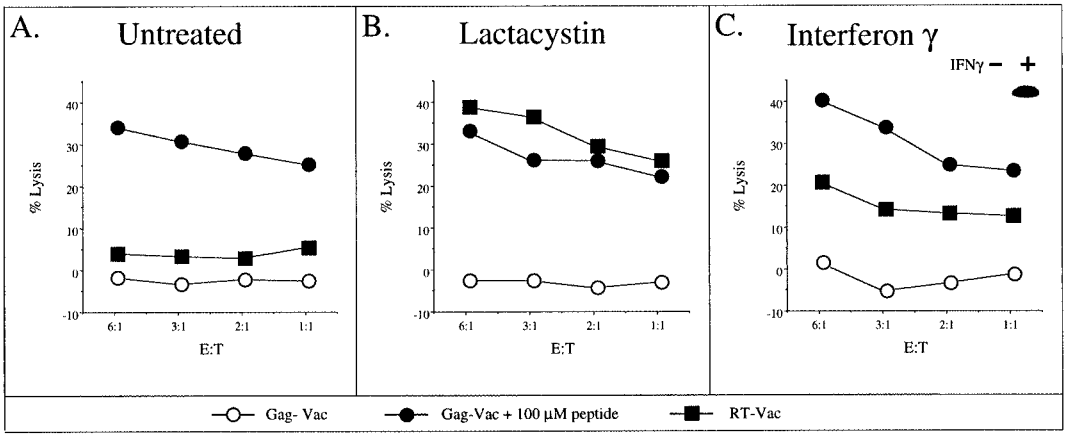

FIGURE 4. IFN-γ exposes a cryptic epitope in HIV1 RT. HeLa cells transfected with HLA A2 and infected with RT-Vac do not present VIYQYMDDL (*A*). Treatment with lactacystin (*B*) or IFN-γ (*C*) restores presentation of this epitope. The inset (*C*) shows IFN-γ treatment massively up-regulates LMP7 expression in these cells.

same effect as treatment with lactacystin in that it increased presentation of the VIYQYMDDL epitope from RT and decreased the lysis of treated cells by CTL specific for the ILKEPVHGV epitope (data not shown). We were unable to inhibit the production of the VIYQYMDDL epitope in lactacystin-treated cells with 10 μM Bestatin (British Biotechnology, Oxford, U.K.), 10 μM E64d, 100 μM Z-GPFL-CHO, 10 μM TPCK/ZPCK, and 10 μM AAF-chloromethyl ketone, which has been reported to inhibit the tricorn protease (26), or 10 μM of the HIV-1 protease inhibitor Ritonavir, which we have shown to inhibit the proteasome (27) (data not shown).

The effect of LMP7 on presentation of the subdominant HIV-1 epitope VIYQYMDDL is of importance since the LMP subunits are IFN-γ inducible. Release of IFN-γ by T lymphocytes during HIV-1 infection may alter Ag-processing pathways to enhance the presentation of cryptic epitopes. To address this possibility, we examined the effect of IFN-γ treatment on the presentation of the VIYQYMDDL epitope from HIV-1 RT. We used HeLa cells transfected with HLA A2 as targets since the level of expression of LMP7 is very low and can be enhanced by treatment with IFN-γ (Fig. 4*C*). After infection with RT-Vac, HeLa A2 cells do not present the VIYQYMDDL epitope at levels sufficient to induce lysis (Fig. 4). Treatment of these cells with either lactacystin or IFN-γ restores presentation (Fig. 4, *B* and *C*). This effect was not observed in .174 TAP cells, indicating that IFN-γ-mediated enhancement of VIYQYMDDL presentation operates through modulation of genetic expression within the region of the chromosome 6 deletion, which includes LMP7, in these cells (Fig. 5).

Discussion

The results presented in Fig. 3 are consistent with there being two different proteolytic cleavage pathways for HIV-1 RT. The C-terminal (ILKEPVHGV) HLA A*0201-restricted epitope in HIV-1 RT appears to fit the "classical" processing scenario since it is made by the proteasome. Conversely, the N-terminal epitope (VIYQYMDDL) appears to be destroyed by this classical pathway. This epitope is presumably produced as a result of an "alternative" degradation pathway. Such a pathway has been previously postulated (28, 29) and recent reports demonstrate the enhancement of a class I epitope by lactacystin treatment (28, 30). The nature of this alternative antigenic degradation pathway remains the subject of debate (26).

In addition to the two epitopes in this study we have examined the processing requirements of a further HLA A2-restricted epitope from HIV-1 p17 matrix (data not shown). Of these three epitopes only one, the ILKEPVHGV epitope in HIV-1 RT, appears to be produced by the lactacystin-inhibited, threonine protease activity of the proteasome. The other epitopes appear to be the product of other distinct protease activities. It cannot be ruled out that activities other than the threonine protease activity of the proteasome (31) are responsible for the presentation of these other

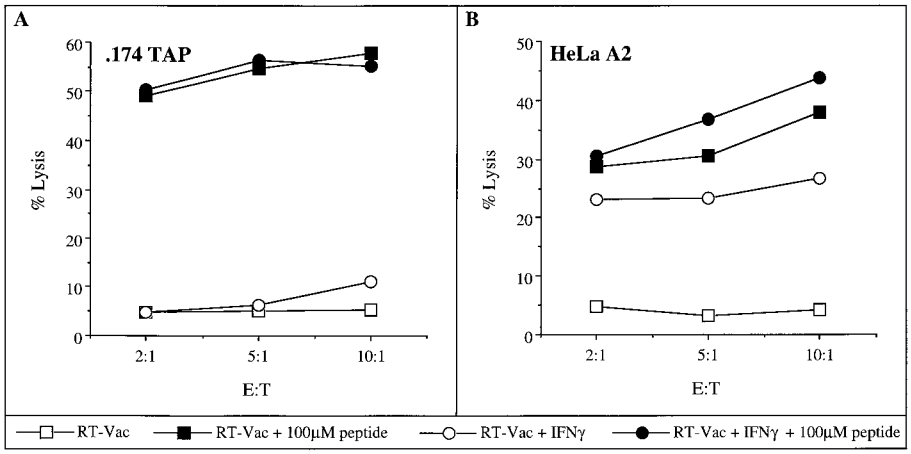

FIGURE 5. IFN-γ induction of VIYQ YMDDL operates through modulation of genetic expression within the region of the chromosome 6 deletion in .174 TAP cells. Treatment of .174 TAP cells with IFN-γ (*A*), at a concentration sufficient to observe an effect in HeLa A2 cells (*B*), does not restore presentation of the VIYQYMDDL epitope after infection with RT-Vac. All data points represent the mean of triplicates.

epitopes. Treatment of cells with lactacystin or overexpression of LMP7 may have pleiotropic effects on cellular metabolism. Thus it is not possible to eliminate the fact that the effects on presentation of the viral Ags studied here are indirect. Furthermore, different cell types may process and present the same CTL epitope through distinct degradation pathways. In this respect, we observed that presentation of the ILKEPVHGV epitope, which is lactacystin-sensitive in the melanoma cell line ME275, is less sensitive to lactacystin treatment in .174 TAP cells.

We document the processing of two HLA A*0201-restricted CTL epitopes in HIV-1 RT by distinct pathways and demonstrate that lack of the IFN-γ-inducible subunit LMP7 impairs the generation of the subdominant epitope. These findings imply that modulation of IFN-γ-inducible subunits of the proteasome during the acute phase of infection may substantially alter the pattern of production of HIV-1 CTL epitopes. It has been suggested that the hierarchy of T cell responses to different epitopes is influenced by the relative efficiency of epitope generation (15, 32). Whether it is these requirements or other factors that determine the dominance of one CTL epitope over another in HIV-1 remains to be determined.

It is tempting to speculate that the release of soluble factors, such as IFN-γ, by early CTL at sites of infection may alter the processing patterns of viral proteins within infected cells. Initial CTL responses may be generated to epitopes produced by the normal Ag processing machinery including the constitutive proteasome. The secretory functions of these CTL may then operate to expose further "cryptic" epitopes within infected tissues.

In addition, our results have some broader implications. Since it is clear that altered protein degradation can lead to the generation of different CTL epitopes, it seems likely that cells altered in their protein degradation may display a different repertoire of self-peptides complexed with MHC class I on their surface. Any such local alteration in protein degradation, perhaps triggered by an infectious agent, may be the basis of some autoimmune conditions.

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