Differential Influence on Cytotoxic T Lymphocyte Epitope Presentation by Controlled Expression of Either Proteasome Immunosubunits or PA28

By Thorbald van Hall,* Alice Sijts,‡ Marcel Camps,* Rienk Offringa,* Cornelis Melief,* Peter-M. Kloetzel,‡ and Ferry Ossendorp*

*From the *Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; and the ‡Institute of Biochemistry, Charité, Humboldt University, 10117 Berlin, Germany*

Abstract

The proteasome is the principal provider of major histocompatibility complex (MHC) class I–presented peptides. Interferon (IFN)-γ induces expression of three catalytically active proteasome subunits (LMP2, LMP7, and MECL-1) and the proteasome-associated activator PA28. These molecules are thought to optimize the generation of MHC class I–presented peptides. However, known information on their contribution in vivo is very limited. Here, we examined the antigen processing of two murine leukemia virus-encoded cytotoxic T lymphocyte (CTL) epitopes in murine cell lines equipped with a tetracycline-controlled, IFN-γ–independent expression system. We thus were able to segregate the role of the immunosubunits from the role of PA28. The presence of either immunosubunits or PA28 did not alter the presentation of a subdominant murine leukemia virus (MuLV)-derived CTL epitope. However, the presentation of the immunodominant MuLV-derived epitope was markedly enhanced upon induction of each of these two sets of genes. Thus, the IFN-γ–inducible proteasome subunits and PA28 can independently enhance antigen presentation of some CTL epitopes. Our data show that tetracycline-regulated expression of PA28 increases CTL epitope generation without affecting the 20S proteasome composition or half-life. The differential effect of these IFN-γ–inducible proteins on MHC class I processing may have a decisive influence on the quality of the CTL immune response.

Key words: antigen processing • major histocompatibility complex class I • immunoproteasomes • PA28 • murine leukemia virus

Introduction

The presentation of peptides in MHC class I molecules is a crucial prerequisite for effective CTL responses. The intracellular processing of these peptides depends on several specialized molecules, each contributing to one of the sequential steps in class I antigen presentation. After generation of peptides by proteolysis, the transporter associated with antigen processing (TAP)[1] molecules transport the precursor peptides into the endoplasmic reticulum, where they can bind to newly formed MHC class I molecules to be transported to the cell surface (for reviews see references 1, 2). MHC class I–bound peptides can be considered side prod-ucts of the continuous turnover and degradation of intracellular proteins. A major proteolytic system is the proteasome, a multicatalytic protease complex responsible for most cytosolic protein breakdown. The proteasome has been designated as the main provider of precursor peptides for MHC class I presentation (3). The core 20S proteasome is a cylindrical structure consisting of four stacked rings, each containing seven homologous but distinct α or β subunits. The several hydrolyzing activities are conferred by the β subunits located in the two inner rings (for review see reference 4).

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[1]*Abbreviations used in this paper:* B6, C57BL/6; env, Moloney MuLV env protein; gag, Moloney MuLV gag protein; gagL, leader sequence of Pr75-gag; LMP, low molecular weight proteins; MEC, mouse embryo cell; MuLV, murine leukemia virus; TAP, transporter associated with antigen processing.

The important immunomodulatory cytokine IFN-γ, produced during an immune response by activated T helper lymphocytes and NK cells, enhances antigen presentation by upregulation of MHC and TAP gene products as well as proteasome subunits and regulators (5). Incubation of vertebrate cells with IFN-γ alters proteasome activity qualitatively by induction of several proteasome-associated molecules (6), thereby recruiting this basal protein degradation system for enhanced presentation of certain immunogenic peptides to the immune system. Three newly expressed proteasome β subunits (LMP2[1], LMP7, and MECL-1) replace the three constitutive β subunits (in mice: δ, MB-1, and MC14, respectively) in the core 20S proteasome. After the recent identification of MC14/ MECL-1 as the third pair of proteasomal subunits (7–9), several groups reported that these so-called immunosubunits are incorporated interdependently. The exchange of one subunit strongly favored the incorporation of another (10). Transfection of LMP2 and/or LMP7 in cell lines is known to alter cleavage specificity of 20S proteasomes (11), predominantly leading to precursor peptides that contain the MHC class I–presented CTL epitopes. Furthermore, targeted deletion of LMP2 and LMP7 in mice results in some immunodeficiency (12, 13). These findings indicate a role for LMP7 and LMP2 in MHC class I antigen processing. However, the novel findings concerning the coordinated incorporation of the three immunosubunits makes it difficult to interpret earlier studies on cleavage specificity in which single subunits were transfected in cell lines. More appropriately, the functional contribution of the three immunosubunits must be studied after expression of the joint subunits in the absence of other IFN-γ–inducible genes.

Besides the proteasome immunosubunits, IFN-γ also regulates the expression of the proteasome-associated complex PA28 (REG or the 11S regulator). PA28 was identified as a proteasome activator that strongly increases the maximal velocity of the hydrolytic reaction and decreases the concentration of substrate required for cleavage by purified 20S proteasomes (14, 15). Isolated PA28 does not display any enzymatic activity by itself. Three homologous PA28 subunits have been cloned thus far, of which only the α and β subunits are strongly induced by IFN-γ (16). Protein chemical analyses have revealed that the α and β subunits form a tightly connected heterohexameric or heteroheptameric complex, composed of comparable amounts of both subunits (17). Although monomers of α subunits were shown to display some proteasome-enhancing activity, heteromultimers appeared to be more stable and effective (18). The heteromultimer is able to bind to the α-rings of the 20S core proteasome, creating bell-shaped cap structures on both ends (5). The mechanism of PA28-mediated enhancement of the intrinsic proteasome activity is presently unknown.

PA28 was implicated in MHC class I antigen processing. In vitro digestion studies with long peptides comprising dominant CTL epitopes showed that purified PA28 can enhance coordinated dual cleavages by the 20S proteasome, leading to augmented epitope liberation (19). Furthermore,

expression of PA28α in mouse fibroblasts increased the sensitivity for lysis by virus-specific CTLs (20). Conflicting data were obtained by other investigators who did not observe any effect of PA28 on antigen presentation (2). Using PA28 β-subunit knockout mice, Preckel et al. (21) showed that PA28 positively affects antigen presentation. However, the assembly of immunoproteasomes in these mice appeared to be impaired. Therefore, it was concluded that PA28 exerts its effect indirectly via immunoproteasome formation.

To distinguish between the effects of PA28 and that of immunoproteasomes, we generated murine cell lines with tightly controlled expression either of the three immunosubunits or of PA28α and β. By using well-defined CTL clones directed against two murine leukemia virus (MuLV)-derived peptides, we show that the presentation of the immunodominant gag-leader epitope is enhanced by the presence of immunosubunits as well as by PA28. Importantly, the effect of PA28 was exerted in the absence of immunosubunits. The presentation of the subdominant epitope was not affected. Our results supply strong evidence for an independent but differential role of these proteasome-related molecules in the generation of CTL epitopes.

Materials and Methods

Cell Lines and Mice. All cell lines are derived from C57BL/6 (B6, H-2b) mice. RMA and TAP2 mutant RMA-S cells are Rauscher MuLV–induced lymphomas. The TAP1 and TAP2 transfectants of RMA-S (rat mtp1[a] and rat mtp2[a], respectively) have been described previously (22). Transfected mouse embryo cell (MEC) lines were derived from a primary culture of embryonic cells of a B6 mouse deficient for p53. LPS blasts were obtained by culturing spleen cells for 3 d with 10 μg/ml LPS (*Escherichia coli* 0111:B4, Difco Laboratories). Ficolled cells were washed and used as targets in CTL assays. The env-specific CTL clone 10B6, generated against Moloney MuLV in a B6.CH-2bm13 mouse, recognizes the SSWDFITV epitope presented by H-2Kb (env; amino acids 189–196) as previously described (23). H-2Db–restricted CTL clone 1 specific for the gag-leader peptide (CCLCLTVFL; amino acids 75–83) was generated against Moloney MuLV in a B6 mouse, as previously described (24). All cell lines were cultured in Iscove's modified Dulbecco's medium (Biowhittaker Europe), supplemented with 8% heat-inactivated FCS (GIBCO BRL), 2 mM L-glutamine (ICN Biomedicals), 100 IU/ml penicillin (Yamanouchi Pharma), and 30 μM 2-ME (Merck) at 37°C in humidified air with 5% CO_2.

B6 mice were bred under specific pathogen–free conditions in the TNO-PG breeding facility. TAP$^{-/-}$ mice were purchased from The Jackson Laboratory (B6/129 TAP$^{-/-}$).

DNA Constructs and Generation of Transfectant Cell Lines. The eukaryotic expression plasmids pTET-splice and pTET-tTAk, containing the tetracycline-regulated transcription activator tTAk, have been described elsewhere (25). Generation of the MEC217 cells expressing inducible levels of the cDNAs of murine (H-2b haplotype) LMP2, LMP7, and MECL-1 was described recently (26). cDNAs of the murine PA28α and β (H-2b haplotype) were cloned into the EcoRI/EcoRI and SalI/EcoRV sites of the pTET splice vector using standard procedures. The PA28αβ transfected cells were established by calcium-phosphate precipita-

tion. In brief, 7.5×10^5 MEC/tTAk cells (clone 29) were plated in 10-cm dishes, transfected with a plasmid mixture consisting of 10 μg pTET-PA28α, 10 μg pTET-PA28β, and 4 μg pLXSP which confers resistance to puromycin, and then diluted in 96-well plates in medium containing 5 μg/ml puromycin, 200 μg/ml hygromycin B (Merck), and 400 ng/ml tetracycline (Sigma-Aldrich). Growing clones were screened for expression of PA28α and PA28β by immunoblot analysis using specific antisera.

Isolation of PA28 Complexes. To isolate PA28 complexes, the pellets of 2×10^7 MEC-PA28 cells grown in the absence or presence of tetracycline for 3 d were lysed in 800 μl of lysis buffer (0.1% Triton X-100, 50 mM Tris-HCl, 5 mM MgCl$_2$, and 1 mM EDTA [pH 7.5]) without protease inhibitors. Cell lysates were freeze-thawed three times and then applied to a 10–40% glycerol gradient that was centrifuged for 16 h at 40,000 rpm in a Sorvall ultracentrifuge. Gradient fractions of 600 μl were collected and tested for the presence of PA28 by Western blot analyses using PA28α- and PA28β-specific polyclonal rabbit antisera (10, 27).

To determine PA28 activity, 20 μl of the glycerol gradient fractions and 80 μl of assay buffer (50 mM Tris-HCl [pH 7.5], 25 mM KCl, 10 mM NaCl, 1 mM dithiothreitol, and 0.1 mM EDTA) containing 100 μM Suc-LLVY-AMC were incubated in 96-well plates. To each well, 30 ng of 20S proteasomes of nontransfected MECs were added and the reactions were incubated for 1 h at 37°C. Fluorescence emission was measured at 460 nm (excitation 355 nm) with a Fluorostar reader.

Isolation of Cellular 20S Proteasomes and Western Blot Analysis. Proteasomes were purified from MEC217 cells cultured in the absence or presence of tetracycline (10 ng/ml and 400 ng/ml) as previously described (26). Protein content in the samples was quantified at an OD of 280 nm. 200 ng of material was separated on 12% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Blots were incubated for 1 h in 10% horse serum/5% (wt/vol) lowfat dry milk/0.4% Tween-20 in PBS and then probed overnight with polyclonal mouse LMP2-, δ-, MECL-1–, MC14-, LMP7-, MB1-, and MC3-specific rabbit antisera at a 1:1,000 dilution in 2% dry milk/0.1% Tween-20 in PBS. Filters were incubated with horseradish peroxidase–conjugated anti–rabbit IgG and developed by enhanced chemiluminescence (Boehringer Mannheim).

Metabolic Labeling, Immunoprecipitation, and Gel Analyses. A total of 2.5×10^5 MEC-PA28 cells were cultured with 400 ng/ml tetracycline or without tetracycline, pulse-labeled for 1 h with 150–300 μCi/ml translabel [^{35}S]methionine, and then chased for different time intervals. Cells were harvested in 1% NP-40 lysis buffer with protease inhibitors and the lysates were cleared by centrifugation for 8 min at 14,000 rpm. 20S proteasomes and PA28α were immunoprecipitated from the lysates using rabbit anti-20S or anti-PA28α antisera and analyzed by SDS-PAGE or by two dimensional NEPHGE/SDS-PAGE, as previously described (26). Gels were exposed for autoradiography. Radioactive signals were quantified using a Molecular Dynamics phosphorImager.

Moloney MuLV Infections. Abelson Moloney virus was derived from NIH/3T3 Abelson virus nonproducer cells (ANN-1), which were productively infected with cloned Moloney MuLV (28). Batches of virus containing supernatants were collected after 24 h and stored at −80°C.

All MEC lines were infected with Abelson Moloney MuLV by culturing for 3–4 d in complete culture medium, containing 50% (vol/vol) virus-containing supernatant with 10 μg/ml polybrene (Sigma-Aldrich). Expression of viral genes was determined by flow cytometry using rabbit FITC-conjugated anti–Moloney

MuLV antiserum. Titrated concentrations of tetracycline were added, starting 1 d before virus infection.

CTL Activation Assays. Cytolytic activity of CTL clones was measured and calculated by ^{51}Cr-release assays, as previously described (24). IFN-γ release by CTLs was measured in U-bottom plates (Costar) using 8×10^3 CTLs incubated with indicated amounts of stimulator cells in the presence of recombinant human IL-2 (5 Cetus units/ml; Eurocetus). Supernatants were harvested after 8–18 h and IFN-γ content was measured by sandwich ELISA technique using anti–mouse IFN-γ–specific antibodies (clones XMG1.2 and biotinylated R4-6A2; PharMingen), as previously described (29).

Treatment with Proteasome Inhibitors. Moloney MuLV–infected target cells were treated with the proteasome inhibitors lactacystin or NLVS (4-hydroxy-5-iodo-3-nitrophenylacetyl-leu-leu-leu-vinylsulfone; NIP-L$_3$VS; Calbiochem) before incubation with CTLs. $1–2 \times 10^6$ cells were incubated with 20 μM lactacystin (Calbiochem) or 20 μM NLVS at 37°C for 4 h. Treatment with proteasome inhibitors did not influence expression of Moloney MuLV–encoded proteins in virus-infected cells, as determined with MuLV-specific antibodies. To control the viability of treated target cells, synthetic peptides (1 μg/ml) were added during the last 30 min of the 4-h incubation period. Inhibitor and unbound peptide were removed by extensive washing before incubation with CTLs.

Results

Processing of the gagL-derived Epitope is TAP and Proteasome Dependent. The role of the immunosubunits LMP2, LMP7, and MECL-1 and of the proteasome regulator PA28 in processing of MHC class I–presented peptides was studied using the well-defined Moloney MuLV system in B6 mice. Two Moloney MuLV–derived CTL epitopes have been described: the immunodominant D^b-binding gag-leader (gagL)–derived peptide CCLCLTVFL (30) and the subdominant K^b-binding gp75-env–derived peptide SSWDFITV (23). In earlier studies we have shown that the presentation of the env-derived epitope depends on the function of TAP (31) and proteasomes (29). The gagL peptide needs to be liberated from the leader sequence of the gag-Pr75 protein. In Moloney MuLV, this leader encodes 88 amino acids and serves to directly translocate the Pr75 precursor protein into the endoplasmic reticulum (32). Here, we examined the necessity of TAP for presentation of the gagL-derived epitope, making use of the TAP2 mutant cell line RMA-S, which lacks a functional TAP1,2 heterodimer. The wild-type RMA and the mutant RMA-S cell lines both express the gag-Pr75 protein. These cell lines were tested for recognition by the gagL-specific CTL clone in a chromium cytotoxicity assay. RMA-S cells were not sensitive to lysis, whereas RMA cells were killed to a high level (Fig. 1 A). Stable transfectants of RMA-S with the rat TAP1 or TAP2 cDNA showed that only transfection of the TAP2 gene resulted in restored sensitivity for lysis by the gagL-specific CTLs. In addition, LPS blasts from wild-type B6 and TAP1$^{-/-}$ mice were infected with Moloney MuLV and tested for recognition by gagL-specific CTLs. Secretion of IFN-γ by the gagL-specific CTLs was markedly lower upon incubation with in-

fected TAP1$^{-/-}$ LPS blasts when compared with infected wild-type LPS blasts (Fig. 1 B), indicating that at least the majority of the gagL peptides need to be transported by TAP before reaching the cell membrane. Also the env-specific CTLs selectively recognized Moloney MuLV–infected wild-type cells (Fig. 1 C).

To investigate the role of proteasomes in the generation of the gagL-derived peptide, we used the proteasome-specific inhibitors lactacystin and vinyl sulfone NLVS. MECs from B6 mice were infected with Moloney MuLV, resulting in recognition by the gagL-specific CTL clone (Fig. 2 A). Preincubation of the infected MECs with the proteasome-specific inhibitors completely abrogated recognition of Moloney-infected target cells by the gagL-specific CTL (Fig. 2 A) and env-specific CTL (data not shown) (29). These inhibitor-treated targets were still able to present synthetic peptide to the CTLs (Fig. 2 B). Thus, the presentation of the CTL epitope from the leader of gag requires functional proteasomes as well as TAP heterodimers.

Enhanced In Vivo Generation of gagL- but not env-derived Peptide by Immunoproteasomes.   The enzymatic activity of 20S proteasomes is mediated by three different β-subunits, δ (or β1), MB1 (or β5), and MC14 (or β2), which are exchanged for the so-called immunosubunits, LMP2 (induced β1; iβ1), LMP7 (iβ5), and MECL-1 (iβ2), respectively, after treatment with IFN-γ. However, IFN-γ induces a wide variety of proteins involved in MHC class I

and class II antigen presentation, including additional proteasome-associated regulatory proteins. To investigate the role of the three proteasome immunosubunits, we used an MEC line (MEC217) with coordinated expression of the murine cDNAs encoding LMP2, LMP7, and MECL-1 driven by a strong tetracycline-regulated promoter (26). When cultured in the presence of high concentrations of tetracycline (400–1,000 ng/ml), these cells express proteasomes with constitutive subunits, comparable to untransfected MECs (data not shown). Lower doses of tetracycline allow defined expression levels of the introduced cDNAs, resulting in a gradual replacement of the constitutive subunits δ, MC14, and MB1 by the immunosubunits LMP2, MECL-1, and LMP7, respectively (Fig. 3). Probing of the α-subunit MC3 showed that equal amounts of 20S proteasomes were loaded in each lane (Fig. 3). Using this system we are able to uncouple the effects of the replacement of the immunosubunits from the multiple IFN-γ effects in vivo.

Using these LMP2-, LMP7-, and MECL-1–transfected MEC217 cells, we tested the efficiency of env- and gagL-derived CTL epitope generation in relation to immunosubunit expression. MEC217 cells were infected with Abelson Moloney virus and cultured in the absence or presence of two concentrations of tetracycline. After 3–4 d, these cells were harvested and used as targets for gagL- and env-specific CTLs in an IFN-γ release assay. As shown in
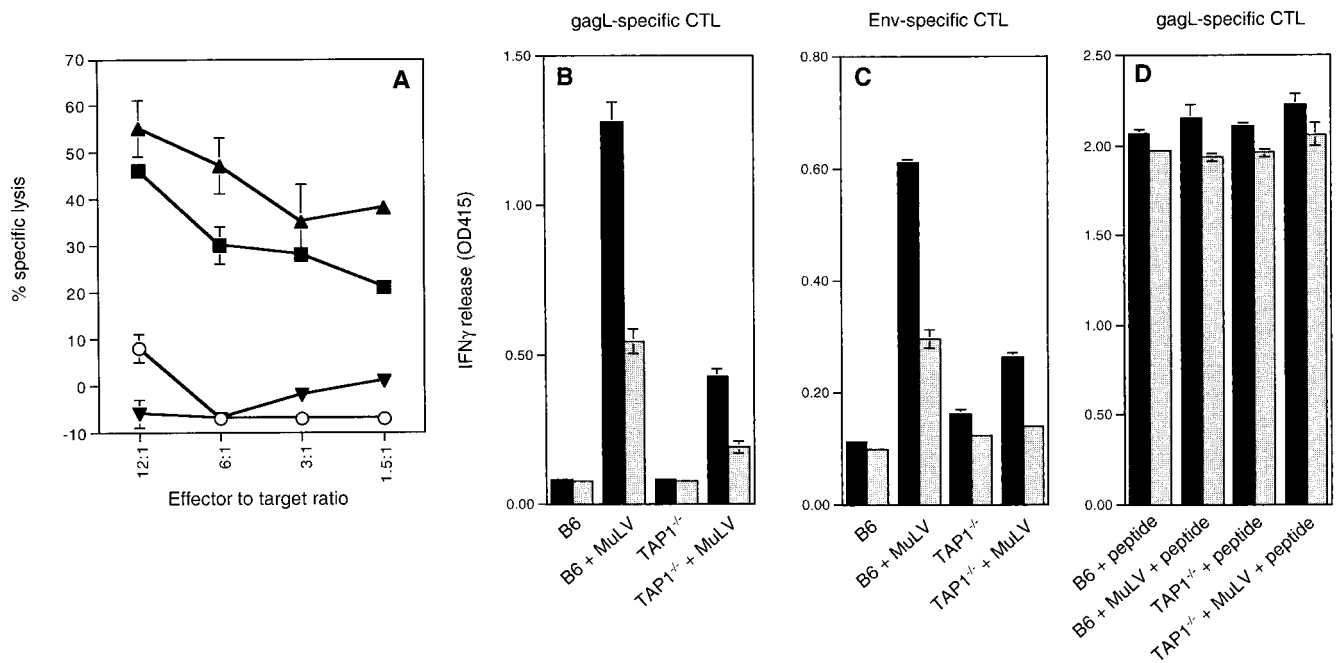

Figure 1.   TAP-dependent presentation of MuLV gagL-derived CTL epitope. (A) Cytolytic activity of a gagL-specific CTL clone against Rauscher MuLV–induced RMA lymphoma and TAP2-mutant RMA-S lymphoma cell lines. RMA-S cells stably transfected with rat cDNA of TAP1 (mtp1ª) or TAP2 (mtp2ª) are indicated as RMA-S.rTAP1 and RMA-S.rTAP2, respectively. CTLs were incubated for 4 h with Cr-labeled target cells at the effector to target ratio as depicted (x-axis). Error bars represent standard deviation of triplicate wells. One representative experiment out of three is shown. ■, RMA; ○, RMA-S; ▼, RMA-S.rTAP1; ▲, RMA-S.rTAP2. LPS blasts derived from wild-type B6 and TAP1$^{-/-}$ spleen cells were infected with Moloney MuLV and incubated with gagL-specific (B) and env-specific (C) CTL clones. 4×10^4 LPS blasts (black bars) and 2×10^4 LPS blasts (gray bars) were incubated with a constant number of CTLs (8×10^3). After 18 h, IFN-γ release was measured with ELISA technique and OD at 415 nm is shown. Recognition of LPS blasts exogenously loaded with synthetic gagL peptide (CCLCLTVFL; 1 μg/ml) is shown in D. Similar results were obtained in an additional experiment. The means of triplicate wells and the standard deviations are shown.
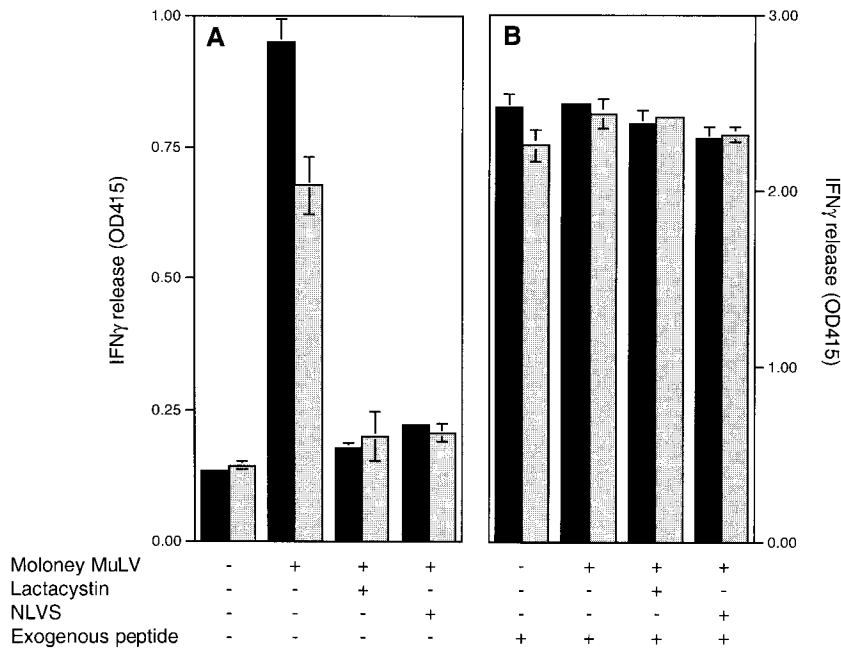
Figure 2. Proteasome-mediated processing of the MuLV gagL epitope. Moloney MuLV infected B6 MEC line was treated with the proteasome inhibitors lactacystin (20 μM) or NLVS (20 μM) for 4 h. After extensive washing, 2×10^4 (black bars) or 5×10^3 target (stippled bars) cells were incubated with the gagL-specific CTL clone (8×10^3) for 8 h (A). Targets were loaded with synthetic peptide during the last 30 min of the treatment with inhibitors to test the peptide-presenting capacity of treated target cells (B). IFN-γ release was measured with ELISA. One representative experiment out of three is shown. Bars represent mean values of triplicate wells ± SD.

Fig. 4 B, env-specific CTLs incubated with induced or noninduced MEC217 cells released intermediate and comparable amounts of IFN-γ, indicating that expression of the immunosubunits did not alter the stimulatory capacity of these cells. Remarkably, the culture of infected MEC217 cells with diminishing concentrations of tetracycline resulted in strongly increased recognition by the gagL-specific CTLs (Fig. 4 A). Since the CTL response was markedly enhanced towards targets with induced expression of the immunosubunits, we concluded that "immunoproteasomes" processed the gagL peptide more efficiently than did constitutive proteasomes. The expression level of cell surface MHC class I molecules was not detectably increased in the induced cells, as measured by flow cytometry. Furthermore, all MEC217 transfectants were equally well recognized by CTLs when exogenously loaded with synthetic peptide, indicating comparable peptide-presenting capacities (data not shown). Treatment of the induced MEC217 cells with the proteasome inhibitors lactacystin or NLVS resulted in strongly impaired CTL recognition, which could again be recovered by exogenous loading with synthetic peptide (Fig. 4 C).

Several control experiments demonstrated that the observed increased recognition could be attributed entirely to the induced immunosubunits. First, a role for tetracycline or the tTAk transcription activator in peptide processing was excluded by testing Moloney-infected control transfectants (data not shown). In addition, immunofluorescence studies with MuLV-specific antibodies showed equal expression levels of viral gag and env proteins on induced and noninduced cells, indicating that the different target cell populations were infected with the same efficiencies (data not shown). In conclusion, the exchange of immunosubunits in the proteasome strongly increased the presentation of the gagL-epitope, but not the env-derived CTL epitope.

Controlled Expression of Functional PA28α/β Complexes Does Not Alter 20S Proteasome Composition. Using the same tetracycline-regulated expression system, we have generated MEC transfectants containing the murine cDNAs of PA28α and β. When these cells were cultured with high concentrations of tetracycline (400–1,000 ng/ml), very low levels of endogenous PA28α and β proteins were detected, comparable to the levels in control transfectants containing the regulatory tTAk encoding plasmid only. After withdrawal of tetracycline, the PA28α and β proteins were strongly upregulated within a few hours, as tested by metabolic labeling. High and stable expression levels were reached after 8–24 h of culture in the absence of tetracycline (data not shown).

To study if the induction of the PA28α and β subunits led to the formation of functional activation complexes, we isolated PA28 from MEC-PA28 cells that were cultured for
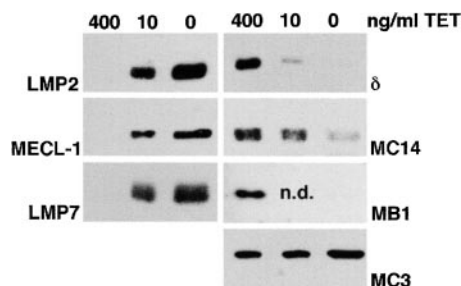

Figure 3. Tetracycline-controlled exchange of constitutive subunits for immunosubunits in 20S proteasomes of MEC217 cells. 20S proteasomes were isolated from MEC217 cells that were cultured in the absence or presence of 10 ng/ml or 400 ng/ml tetracycline for 3 d. 200 ng of purified 20S proteasomes were separated by SDS-PAGE and probed with polyclonal rabbit antisera specific for the immunosubunits LMP2, MECL-1, and LMP7, for the constitutive β-subunits δ, MC14, and MB1 and for the α-subunit MC3, as indicated. n.d., not determined.
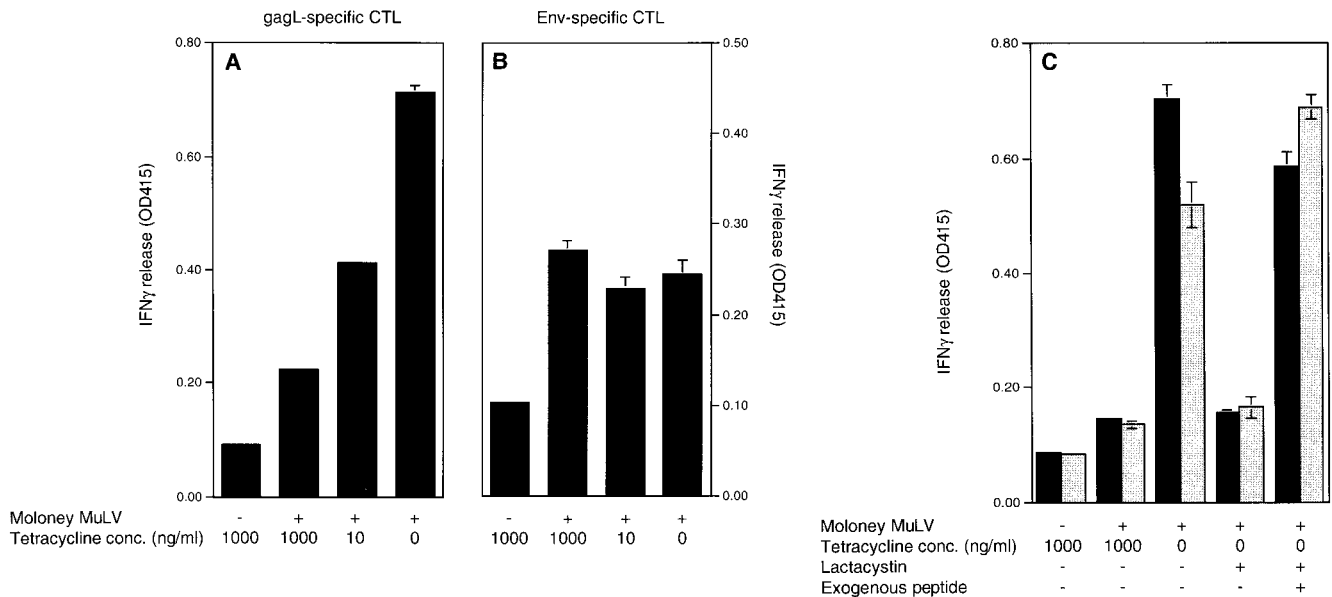
Figure 4. Expression of the immunosubunits enhances presentation of the MuLV gagL, but not the env CTL epitope. The mouse embryo cell line MEC217 containing the inducible immunosubunits LMP2, LMP7, and MECL-1 was infected with Moloney MuLV and cultured with decreasing concentrations of tetracycline for 3 d. Infected and noninfected control cells were harvested and used as targets (2×10^4 cells) with gagL-specific (A) and env-specific (B) CTL clones. IFN-γ release after 9 h of incubation is shown. Mean values of duplicate wells are shown. Moloney MuLV–infected MEC217 cells cultured for 3 d without tetracycline to induce the incorporation of the immunosubunits were treated for 4 h with 20 μM proteasome inhibitor lactacystin (C). Targets cells were extensively washed and 2×10^4 (black bars) or 1×10^4 (stippled bars) cells were incubated with gagL-specific CTL (8×10^3). Representative example of four different experiments with similar outcomes is shown. Mean values of triplicate wells $\pm$ SD are shown.

3 d with high concentrations (500 ng/ml) of tetracycline or without tetracycline. Lysates of these cells were separated on a glycerol gradient and fractions of 600 μl were collected and assayed for the presence of PA28 by immunoblot analysis. After this protocol, assembled PA28 complexes are expected to migrate in fractions 7–10 of the glycerol gradient. In the noninduced cells, PA28β was undetectable and only low levels of PA28α were detected (Fig. 5, A and B), indicating low expression levels of endogenous PA28 in B6 MECs. In contrast, strong signals for PA28α and β were found in fractions 7–10 of induced MEC-PA28 cells, showing induced expression of PA28 and proper assembly into hexa- or heptameric complexes (Fig. 5, A and B). The PA28 that is present in the early fractions most likely consists of nonassembled single subunits.

The activity of the PA28-containing fractions was established by measuring the capacity to enhance the 20S proteasome-mediated cleavage of the Suc-LLVY-AMC substrate. As expected, in the absence of 20S particles, the PA28-containing fractions (7–10) did not show any enzymatic activity (Fig. 5 D). However, when low amounts of purified constitutive 20S proteasomes were added to each fraction, a clear enhancement of substrate conversion was observed in the presence of fractions that contained multimeric PA28 (Fig. 5 C). The same glycerol fractions of noninduced MEC-PA28 cells were markedly less efficient in enhancing 20S activity. The enzymatic conversion that was observed in the higher fractions was probably due to the presence of isolated endogenous 20S, 26S, or even PA28-capped 20S proteasomes (fractions 12–20; reference 33).

Thus, the presence of PA28 strongly enhances the hydrolysis of the Suc-LLVY-AMC substrate, showing that PA28 induced in our MEC-PA28 transfectant is functional.

Recently, PA28 was postulated to be crucially involved in the assembly of immunoproteasomes (21). To examine the potential effects of PA28 on proteasome subunit composition in our MEC-PA28 transfectant, induced and noninduced MEC-PA28 cells were metabolically labeled and 20S complexes were immunoprecipitated (Fig. 6). Two-dimensional gel analysis revealed that 20S proteasomes of both PA28-induced and noninduced cells contained the constitutive β-subunits (δ and MB1), but lacked the immunosubunits (LMP2, LMP7, and MECL-1), indicating the sole formation of constitutive proteasomes in these cells (Fig. 6 A). Furthermore, pulse–chase analyses showed that PA28 affected neither 20S proteasome half-life (Fig. 6 B) nor the total amount of 20S proteasomes, as evident from the comparison of the obtained radioactive signals with those of an immunoprecipitated cellular protein (data not shown). Thus, expression of PA28α/β complexes does not alter the quality or quantity of 20S proteasomes in MEC-PA28 cells. In conclusion, the tetracycline-inducible system enables us to exclusively study the role of PA28 on CTL epitope presentation in the absence of other changes in the proteasome.

Expression of PA28α/β Enhances Presentation of gagL- but not of env-derived CTL Epitopes. To examine the effect of PA28α/β on presentation of the MuLV-encoded CTL epitopes, we used Abelson Moloney virus–infected MEC-PA28 cells in CTL activation assays. Virus-infected MEC-
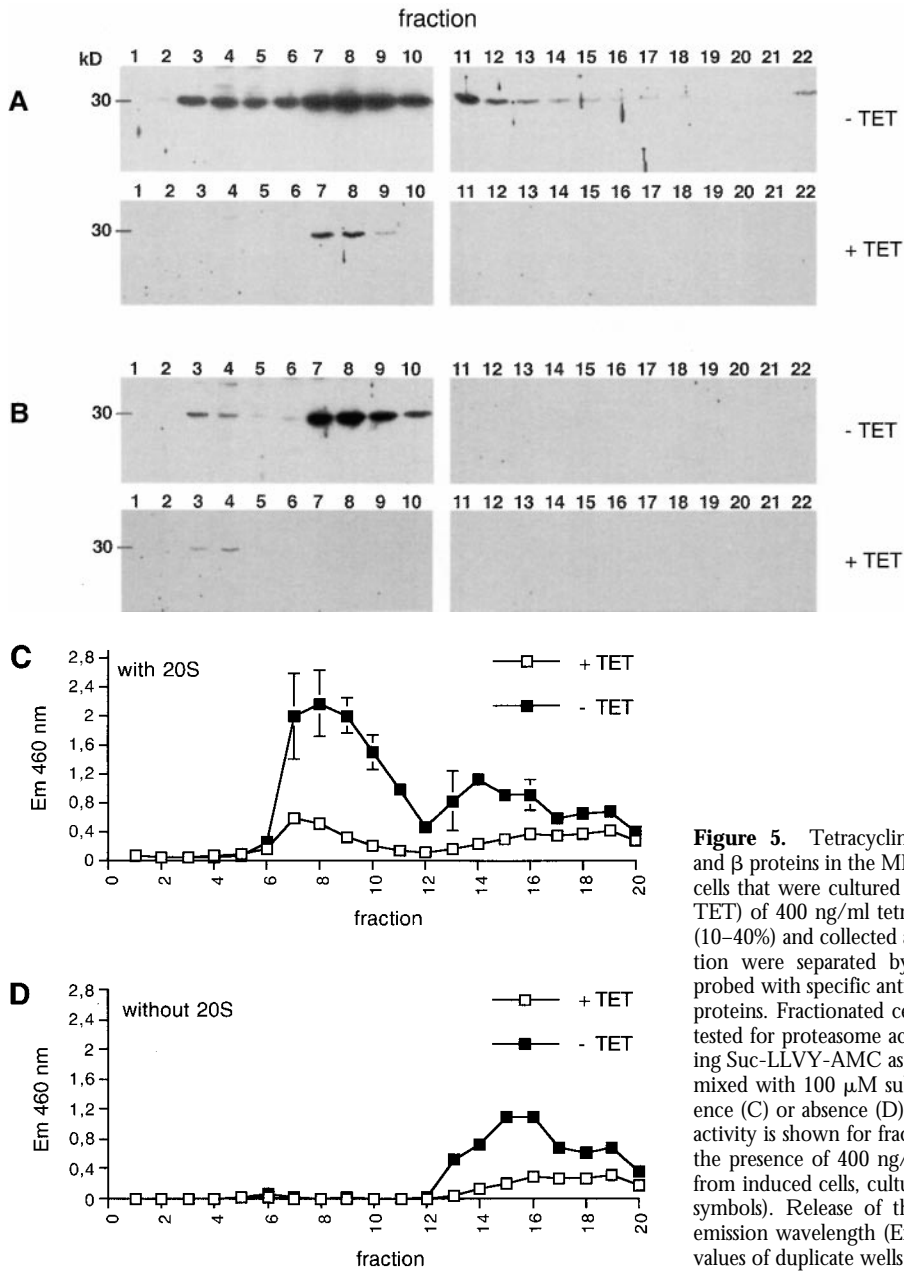
fraction

A   - TET

+ TET

B   - TET

+ TET

Figure 5. Tetracycline-regulated expression and function of PA28α and β proteins in the MEC-PA28 B6 cell line. Cell lysates of MEC-PA28 cells that were cultured for 3 d in the absence (− TET) or presence (+ TET) of 400 ng/ml tetracycline were centrifuged on a glycerol gradient (10–40%) and collected as 600 μl fractions. Samples of 25 μl of each fraction were separated by SDS-PAGE, blotted onto nitrocellulose and probed with specific antibodies to detect the PA28 α (A) and PA28 β (B) proteins. Fractionated cell lysates of MEC-PA28 cells were subsequently tested for proteasome activating capacity in a peptide hydrolysis assay using Suc-LLVY-AMC as a substrate. Of each glycerol fraction, 20 μl were mixed with 100 μM substrate in assay buffer and incubated in the presence (C) or absence (D) of 30 ng purified 20S proteasomes. Hydrolyzing activity is shown for fractions isolated from noninduced cells, cultured in the presence of 400 ng/ml tetracycline (open symbols) and for fractions from induced cells, cultured in the absence of tetracycline for 3 d (closed symbols). Release of the fluorescent AMC-group was detected at an emission wavelength (Em) of 460 nm using a spectrofluorometer. Mean values of duplicate wells are shown.

PA28 cells were cultured for 3–4 d in the presence or absence of tetracycline. As shown in Fig. 7 A, the recognition of induced cells by the gagL-specific CTL clone was markedly higher than recognition of noninduced cells. Three independent experiments showed that optimal expression of PA28 increased the MHC class I presentation of the gagL peptide between 60 and 100%. Suboptimal induction at intermediate tetracycline concentrations (10 ng/ml) resulted in intermediate CTL activation (Fig. 7 A). Analysis of other secreted cytokines, such as TNF-α, reflected the results obtained for IFN-γ. Importantly, the addition of IFN-γ–neutralizing antibodies during the assay did not alter the obtained results, indicating that the IFN-γ produced by the CTLs did not influence the presentation of

the epitope (data not shown). In contrast to the gagL peptide, presentation of the env-derived CTL epitope was not influenced by the presence of PA28 (Fig. 7 B). Since we found that the response of the env-specific CTLs was also not increased after induction of the immunosubunits (Fig. 4), we performed control experiments to exclude the possibility that the initial amount of presented env-peptide was already saturating for the CTL clone. Addition of a small number of peptide-loaded target cells to Moloney-infected targets strongly increased the IFN-γ responses of the CTLs, indicating that the presented amounts of env-peptide in Moloney-infected cells was not saturating (data not shown).

To establish whether the stimulating effect of PA28 on the epitope generation was mediated via the proteasome,
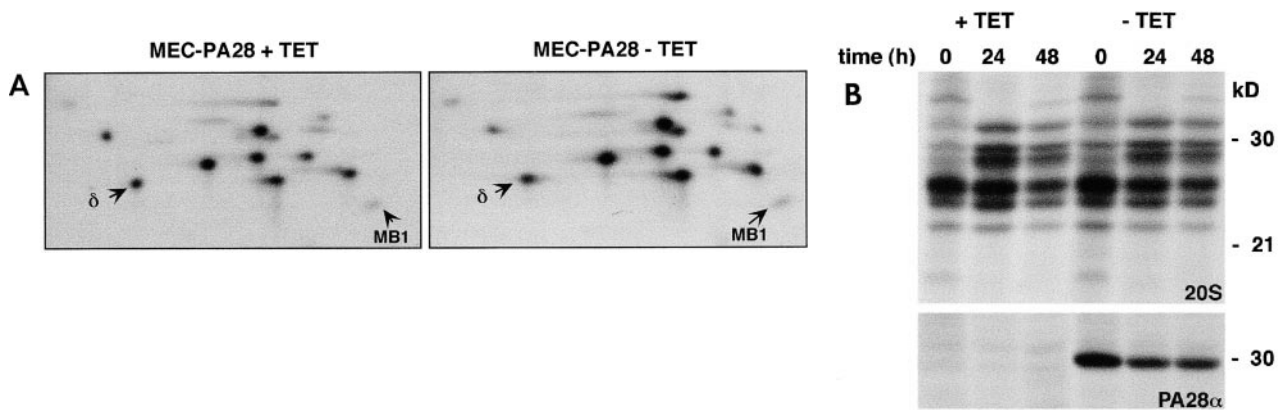
Figure 6. PA28 does not influence 20S proteasome subunit composition or stability. MEC-PA28 cells were cultured in the presence (400 ng/ml) or absence of tetracycline, pulsed with [^{35}S]methionine, and chased for 8 h (A). 20S proteasomes were immunoprecipitated from the detergent lysates and subjected to two-dimensional gel electrophoresis. In B, labeled MEC-PA28 cells were chased for 0, 24, and 48 h. 20S proteasomes and PA28α were immunoprecipitated and separated by SDS-PAGE. Molecular weight markers in kD are indicated on the right. Signals at t = 0 h represent 20S precursor subunits (top).

we inhibited the proteasome by preincubation with lactacystin. In this independent experiment, lactacystin treatment rendered the MEC-PA28 cells completely unstimulatory for the gagL-specific CTL and the env-specific CTL (Fig. 8, A and B). This indicated that the stimulating effect of PA28 on the presentation of the gagL-epitope is mediated via the proteasome and that this effect is completely dependent on functional proteasome activity. The tetracycline-regulated expression of both PA28α and β genes has enabled us to selectively address the role of PA28 on the processing of CTL epitopes, independently of other IFN-γ–inducible proteins. These data suggest that PA28 associates with constitutive proteasomes in a functional way, leading to increased CTL epitope generation.

In conclusion, we show evidence for involvement of the proteasome-activator PA28 in the generation of a naturally processed CTL epitope. Induction of PA28 as well as the

proteasome immunosubunits enhanced the presentation of the gagL peptide, showing that these IFN-γ–inducible genes contribute to improved generation of this immunodominant CTL epitope in vivo.

Discussion

Here, we provide evidence for a differential role of both the proteasome-associated immunosubunits and the PA28 activator on the generation of virally encoded CTL epitopes. Although a strong augmentation in epitope processing was determined for one Moloney MuLV–derived CTL epitope, the generation of another Moloney-derived epitope was not influenced by induction of the immunosubunits or PA28 in the same infected cell. Using a tetracycline-regulated transfection system, the expression of LMP2, LMP7, and MECL-1 in one cell line and PA28α
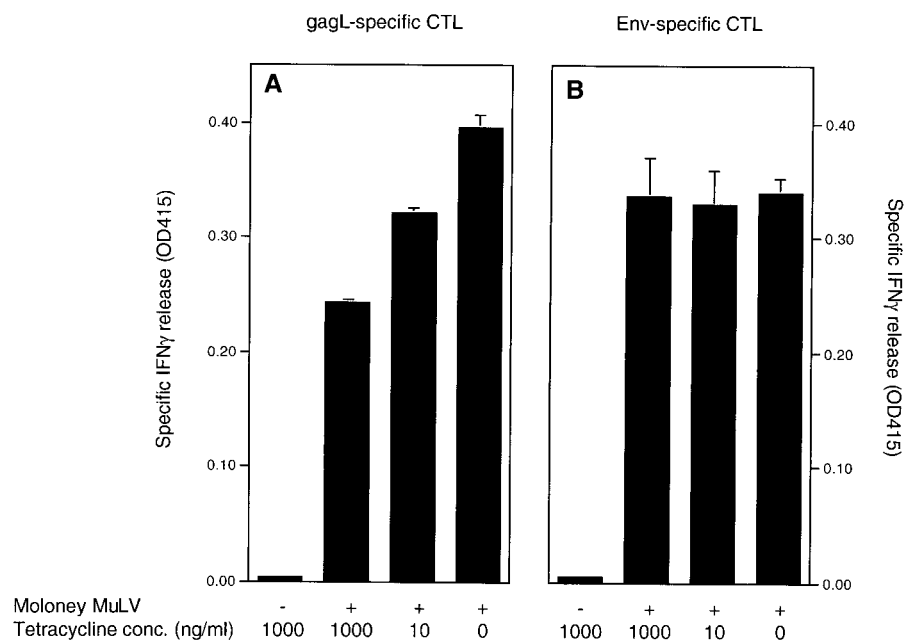

Figure 7. Induction of PA28 selectively increases the presentation of the gagL-derived epitope. Moloney MuLV–infected MEC-PA28 cells were cultured with decreasing concentrations of tetracycline for 3 d and used as targets (2 × 10⁴) with gagL-specific (A) and env-specific (B) CTLs (8 × 10³). IFN-γ release by the CTLs was measured after 9 h. Similar results were obtained in two additional experiments (see also Fig. 7). The mean of duplicate values is shown.
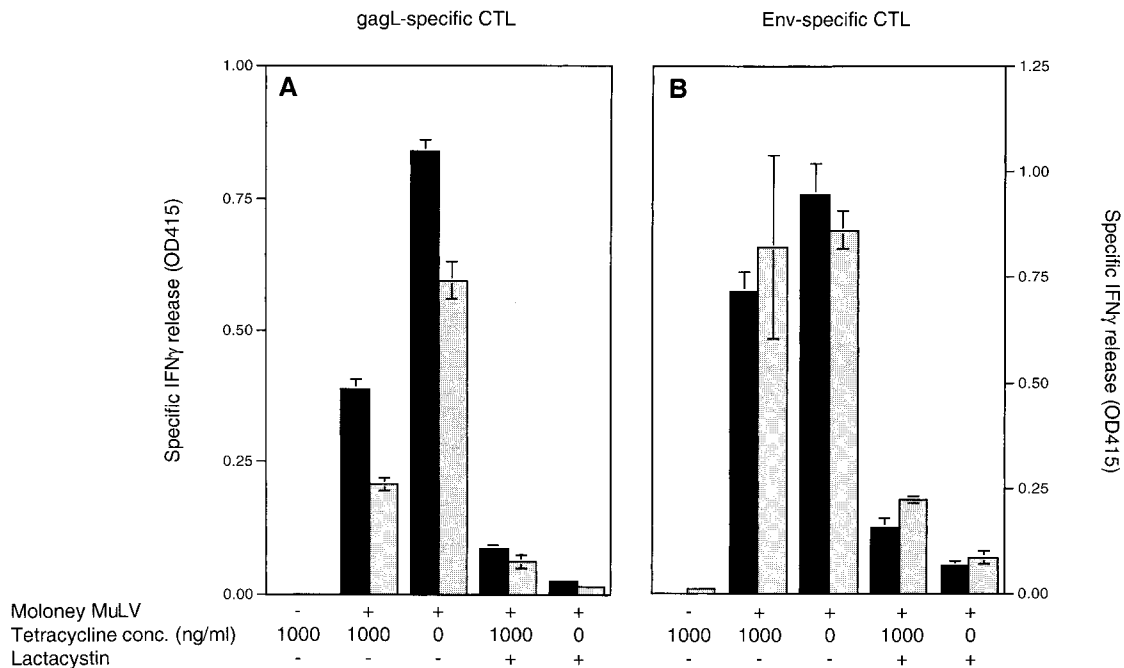
gagL-specific CTL   Env-specific CTL

Moloney MuLV	-	+	+	+	+	-	+	+	+	+
Tetracycline conc. (ng/ml)	1000	1000	0	1000	0	1000	1000	0	1000	0
Lactacystin	-	-	-	+	+	-	-	-	+	+

Figure 8. Enhanced CTL epitope presentation by PA28 is proteasome dependent. Moloney MuLV–infected MEC-PA28 cells were treated with the proteasome inhibitor lactacystin (20 μM) for 4 h. Treated cells were incubated with MuLV gagL-specific (A) and env-specific (B) CTL clones (8×10^3), and IFN-γ release was measured after 18 h. Black bars represent 4×10^4 targets and stippled bars represent 2×10^4 targets. The mean of triplicates ± SD is depicted.

and β in another could be tightly regulated by concentrations of tetracycline in the culture medium. This enabled us to study the effect of these IFN-γ–inducible proteins independently of other IFN-γ–mediated effects. These data suggest that immunosubunits and PA28 increase the presentation of certain but not all CTL epitopes. This, together with the fact that a basal level of epitope presentation was detected in the absence of these molecules, supports the idea that immunosubunits and PA28 are not essential for MHC class I antigen processing in general (34, 35). Rather, subtle changes in cleavage fine specificity or accelerated peptide generation seem to be the major role for the studied IFN-γ–inducible proteasome-related proteins.

Interestingly, the immunosubunits and PA28 only affected the generation of the immunodominant gagL-derived epitope and not the subdominant env-epitope. Next to other factors, proteasome-mediated proteolysis of MHC class I–presented peptides has been shown to influence the hierarchy of OVA peptide–specific T cell responses (36). The subdominant env-derived CTL epitope binds to H-2K[b] with high affinity (23), is efficiently translocated by TAP (37), and has a COOH terminus that is properly cleaved by proteasomes (29). Still, the CTL response towards this peptide in B6 mice is inferior to the response towards the gagL epitope (23). We now observe a correlation between the subdominance of the env-derived CTL epitope and its failure to show increased peptide presentation after induction of either immunosubunits or PA28.

Our findings that the processing of a leader-encoded epitope is dependent on TAP and proteasome were rather unexpected. Apparently, gagL precursor peptides are eventually derived from the cytosol, although the gag-Pr75 molecule, most likely carrying the leader as the transmembrane region, is naturally routed to the cell surface. Alternatively, gagL-epitopes may derive from misfolded gag-Pr75 proteins that are reimported from the endoplasmic reticulum to the cytosol and are targeted for rapid degradation by proteasomes (38). TAP and proteasome-dependent processing were also observed for the D[b]-restricted CTL epitope gp33 of lymphocytic choriomeningitis virus (LCMV) that is encoded on the leader of gp (39, 40). These findings show that leader-derived epitopes can be processed by classical MHC class I processing routes.

Recently, several groups have shown that under physiological conditions the three catalytic subunits LMP2, LMP7, and MECL-1 are exchanged coordinately for the constitutive β subunits in proteasomes. Since the third pair of IFN-γ–regulated subunits MECL-1/MC14 was only recently identified, most studies have not evaluated the role of the three immunosubunits together. Moreover, documentation on the effect of immunosubunits on the generation of CTL epitopes in vivo is very limited. Next to the gagL-peptide of Moloney MuLV we also observed improved presentation of an Adenovirus type 5 E1B-derived CTL epitope due to induced expression of immunoproteasomes in our tetracycline-regulated MECs (26). Strikingly, in the same experiments no differences were detected in the presentation of a CTL epitope from the Adenovirus E1A-protein (Sijts, A., and P.-M. Kloetzel, unpublished observations). In addition, we recently showed that the hepatitis B virus core antigen–derived HBcAg141-151

epitope is produced by immunoproteasomes but not by constitutive proteasomes. Consequently, this epitope was only presented after IFN-γ–induced expression of immunosubunits (41). Thus, these data indicate that immunoproteasomes promote the generation of some MHC class I–presented peptides that are poorly generated otherwise. The recent insight in the interdependent incorporation of the three immunosubunits validated the evaluation of single LMP2- and LMP7-deficient mice, because both knockout mice lack immunoproteasomes. These mice displayed impaired presentation of influenza NP peptide and HY antigen, respectively, whereas no influence was found on the presentation of the OVA-derived peptide (12, 13).

The role of PA28 in antigen processing is currently debated. Although it was originally described as a potential and strong activator of the proteasome in peptide digestion studies, only one study showed some influence of PA28α on the generation of two viral peptides in vivo (20). In addition, we show the importance of PA28 in the generation of some, but not all, CTL epitopes, such as the Moloney MuLV env epitope. In summary, these data on the effects of the IFN-γ–inducible molecules on peptide presentation are observed in several distinct antigenic systems and confirm the interpretation of the results presented here. Importantly, the current study unequivocally documents for the first time independent enhancement of CTL epitope presentation by either the LMP2, LMP7, and MECL-1 subunits, or by the PA28α/β molecules.

A recent study suggests that the action of the PA28α/β complex resides in the induced assembly of immunoproteasomes (42). However, our experiments indicate that PA28 has a separate, distinct mode of action in conjuncture with constitutive proteasomes, independent of immunoproteasomes. Intriguing questions remain, in particular how the immunosubunits and PA28 facilitate the presentation of peptides and what mechanism underlies the differential enhancement in peptide presentation. The presence of immunosubunits might lead to altered cleavage site specificity, depending on the primary amino acid sequence of the substrate and thus may produce MHC class I ligands that are not produced otherwise. However, as with the peptides shown here and probably the majority of epitopes, MHC class I ligands are generated already in the absence of immunosubunits at different efficiencies. Thus, in the case of the gagL-derived epitope analyzed here, it appears that the immunosubunits do not change the specificity of the cleavage sites used, but rather the cleavage site preference of the proteasome (6), resulting in improved liberation of the epitope from the substrate. The differential effect observed for our epitopes may be explained by the different sequence characteristics of the epitopes, including differences in flanking residues and within the epitopes themselves.

In our transfected MEC line, PA28 associates with constitutive proteasomes. In IFN-γ–treated cells, one would expect PA28 to associate with both immunoproteasomes and constitutive proteasomes due to the presence of a mixed population of proteasomes. However, in vitro data have shown that PA28 exerts the same effects on both types

of proteasomes (Kuckelkorn, U., and P.-M. Kloetzel, personal communication). For a number of substrates it has been shown in vitro that PA28 enhances the generation of peptides that are the products of two consecutive endoproteolytic cleavage events (19), possibly due to enhanced proteasomal activity. Although PA28 enhances proteasome activity in vitro, there is no evidence that the expression of PA28 alters the turnover of cellular proteins (20) and thus it appears unlikely that the PA28-mediated effect is due to increased substrate turnover. Indeed, an increase in dual cleavage events would also occur if a substrate is retained longer with the proteasome barrel before release. Therefore, PA28 may control the exit of an antigenic product.

Since most proteins in the cell are turned over via the 26S proteasome complex, the ubiquitin-26S proteasome system is most likely rate limiting with regard to the turnover rate of a given substrate. This raises the question concerning the connection between the PA28-20S proteasome system and the PA700-proteasome (26S proteasome) system within the cell. Based on the recent observation by Hendil et al. (42), we would presently favor a model that combines the ubiquitin-proteasome system and PA28, suggesting that upon induction of PA28 synthesis, PA700–20S immunoproteasome–PA28 complexes are formed. Such a complex would allow the ubiquitin-dependent, rate-controlled turnover of proteins and at the same time guarantee the efficient generation of MHC class I ligands.

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