

Inactivation of a Defined Active Site in the Mouse 20S Proteasome Complex Enhances Major Histocompatibility Complex Class I Antigen Presentation of a Murine Cytomegalovirus Protein

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Summary

Proteasomes generate peptides bound by major histocompatibility complex (MHC) class I molecules. Avoiding proteasome inhibitors, which in most cases do not distinguish between individual active sites within the cell, we used a molecular genetic approach that allowed for the first time the *in vivo* analysis of defined proteasomal active sites with regard to their significance for antigen processing. Functional elimination of the δ /low molecular weight protein (LMP) 2 sites by substitution with a mutated inactive LMP2 T1A subunit results in reduced cell surface expression of the MHC class I H-2L^d and H-2D^d molecules. Surface levels of H-2L^d and H-2D^d molecules were restored by external loading with peptides. However, as a result of the active site mutation, MHC class I presentation of a 9-mer peptide derived from a protein of murine cytomegalovirus was enhanced about three- to fivefold. Our experiments provide evidence that the δ /LMP2 active site elimination limits the processing and presentation of several peptides, but may be, nonetheless, beneficial for the generation and presentation of others.

Key words: proteasome • antigen processing • mutation • active site • low molecular weight protein 2 T1A

Using proteasome-specific inhibitors, the proteasome system has been shown to be involved in antigen processing and to represent the major source for the generation of MHC class I peptides (1–4). The 20S proteasome is an NH₂-terminal nucleophile hydrolase possessing an active site threonine residue (5). It is a cylinder-shaped particle composed of four stacked rings of seven subunits each. In eukaryotes, the seven different α type subunits occupy positions in the two outer rings, whereas the two inner rings are formed by seven different β type subunits (6). The proteolytic activity is restricted to the lumen of the cylinder and is mediated by three of the seven β type subunits, i.e., subunits δ (β 1), MB1 (β 5), and Z (β 2) (parentheses, new nomenclature according to Groll et al., reference 7). Therefore, in total, the 20S proteasome complex possesses six active sites within the two inner β rings. By induction with the cytokine IFN- γ , the active site bearing constitutive β subunits are replaced by their IFN- γ -inducible counterparts low molecular weight protein 2 (LMP2)¹ (i β 1), LMP7 (i β 5), and MECL-1 (i β 2) during proteasome assembly (3, 8, 9).

Of these, LMP2 (i β 1) and LMP7 (i β 5) are encoded within the MHC class II region in the direct neighborhood of the TAP1 and TAP2 peptide transporter genes (10, 11). MECL-1 (i β 2) is encoded outside the MHC locus, but its incorporation into the 20S proteasome complex is guaranteed through the presence of LMP2 (i β 1; reference 12). The IFN- γ -induced replacement of subunit δ (β 1) by LMP2 (i β 1), subunit MB1 (β 5) by LMP7 (i β 5), and Z (β 2) by MECL-1 (i β 2) results in changes of the hydrolytic activities as monitored with short fluorogenic peptide substrates (13, 14). In addition, the incorporation of these subunits strongly alters the cleavage site preferences of the 20S proteasome *in vitro* (14, 15). As a consequence, a different set of peptide products is generated by the 20S proteasome. Under physiological conditions, the ratio between constitutive and cytokine-modified proteasomes complexes changes only slowly. Accordingly, the abundance of certain peptide products as well as their quality will gradually change during the time course of IFN- γ induction. Indeed, targeted deletion of LMP2 (i β 1) and LMP7 (i β 5) in mice caused alterations in antigen presentation, emphasizing the importance of these subunits for the generation of at least certain MHC class I antigens (16, 17). Using proteasome

¹Abbreviations used in this paper: LMP, low molecular weight protein; MCA, amido-4-methylcoumarin; MCMV, murine CMV.

inhibitors, it has been shown that the inhibition of some of the proteasomal peptidase activities affects the processing of MHC class I antigens. (1, 18). However, there exists little active site specificity of the available proteasome aldehyde inhibitors. Even the active site specificity of lactacystin demonstrated *in vitro* is difficult to control in cell experiments since, depending on the experimental condition, lactacystin affects more than one type of active site (19, 20). Therefore, experimental setups using proteasome inhibitors in most cases do not allow one to draw any conclusions on the functional importance of a specific active site for the generation of a defined MHC class I antigen. Such knowledge is, however, important to better understand the basic rules of antigen processing and to develop strategies that may allow either up- or downregulation of the generation of a defined antigenic peptide.

To overcome these problems, we made use of a recently described mutation in the nonconstitutive LMP2 ($\beta 1$) subunit in which the NH_2 -terminal active site threonine was replaced by alanine (21). This T1A mutation resulted in the impairment of correct maturation by autocatalytic processing of the subunit and rendered an proteolytically inactive LMP2 subunit. In this study, we used the inactive mutant to study the functional importance of the δ /LMP2 ($\beta 1$ / $\beta 1$) active sites with regard to MHC class I antigen presentation. Overexpression of the mutant LMP2 T1A subunit in mouse fibroblast cells resulted in an effective replacement of the proteolytically active δ ($\beta 1$) subunit. As a consequence, the mutant LMP2 T1A cells contain proteasomes in which two of the six active sites of the 20S proteasome complex are eliminated. Our experiments demonstrate that the deletion of these active sites generally limits the production of peptides bound to H-2L^d and H-2D^d molecules. At the same time, the mutation enhances the generation and presentation of an H-2L^d epitope derived from the cytomegalovirus pp89 protein.

Materials and Methods

Cell Lines. The BALB/c-derived mouse fibroblast cell lines C4 and B8 were used. The B8 cell line, which is derived from the C4 cells, constitutively expresses the IE I pp89 of the murine cytomegalovirus (22). The B8 cell line was subcloned by limiting dilution and one clone was chosen as recipient for the transfection experiments. The generation of the cDNA constructs of LMP2 and LMP2 T1A, transfection by conventional calcium phosphate precipitation and selection are described in detail in reference 21.

Purification of 20S Proteasomes and Assay of Proteolytic Activity. 20S proteasomes were purified using standard procedures (21). V_{max} and K_m values were determined using the fluorogenic peptide substrates Bz-Val-Gly-Arg-7 Amido-4-methylcoumarin (MCA), Z-Gly-Gly-Leu-MAC, Suc-Leu-Leu-Val-Tyr-MCA, and Methoxysuccinyl-Gly-Leu-Phe-MCA (Bachem, Heidelberg, Germany). The peptides were used in concentrations from 5 to 200 μM and incubated with 1 $\mu\text{g}/\text{ml}$ proteasome in 200 μl of 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 10 mM NaCl, and 0.1 mM EDTA (assay buffer) for 1 h at 37°C as described before (14). Fluorescence intensity was measured at an excitation wave length of 390 nm and an emission wave length of 460 nm in a SLT Fluostar spectrofluorome-

ter. Data were analyzed according to Lineweaver and Burk. All assays were performed in triplicate and repeated three times.

Western Blotting. 15 ng of purified proteasomes were separated by SDS-PAGE and blotted as described (21). The blots were incubated with either LMP2- or δ -reactive antisera. Immunoreactive proteins were detected by enhanced chemiluminescence.

Northern Analysis. The poly A⁺ mRNA of the cell lines C4, B8, B8-LMP2, control, and B8-LMP2 T1A was prepared using commercially available kits (Quiagen, Darmstadt, Germany). 3 μg of mRNA was applied to each lane. Agarose gel electrophoresis, blotting, labeling of the cDNA fragments, and hybridization was performed according to standard procedures (23). We used three different cDNA fragments simultaneously, a PstI/HindIII fragment (bp 297–815), a HindIII/PstI fragment (bp 815–1,314), and a PstI to 3' fragment (bp 1,314–1,788). After hybridization and washing the blot was quantitated and visualized with a Phosphor-Imager (Molecular Dynamics, Krefeld, Germany).

Flow Cytometry. Cells were removed from culture dishes with calcium- and magnesium-free medium, washed, and stained according to standard protocols with the monoclonal antibodies 19/191 (anti-H-2D^d), 3-25.4 (anti-H-2D^d; PharMingen, San Diego, CA), 28-14-8S (anti-H-2L^d), 28-14-8 (anti-H-2L^d; PharMingen), and 15-5-5S (anti-H-2K^d), and a sheep anti-mouse F(ab)2-FITC conjugate as a second-stage reagent. The analysis was performed with a FACSCAN[®] flow cytometer and LYSIS II[™] software (Becton Dickinson, Heidelberg, Germany).

Acid Elution of Natural Peptides and MHC Class I Peptide Binding Assay. For peptide extraction and external-loading peptides we followed the procedure as described previously (24, 25). B8 cells (2×10^9) were separated from the culture dishes with calcium- and magnesium-free medium and washed with PBS to remove serum proteins. The cells were incubated for 15 min on ice in 0.1% TFA/H₂O, sonicated, and kept on ice for another 15 min. Cells were centrifuged for 15 min at 15,000 rpm and the supernatant was collected. High molecular mass material was removed by centrifugation at 4°C through a 10-kD Centricon filter (Amicon Corp., Easton, TX). The peptides were concentrated by Speed Vac centrifugation to a final concentration of ~5 mg/ml as judged by OD 280. B8 cells and the transfectants were cultured for 18 h at 27°C in the presence of either 25 $\mu\text{g}/\text{ml}$ of the synthetic peptide YPHFMPTNL (H-2L^d epitope of pp89) or ~25 $\mu\text{g}/\text{ml}$ of peptides extracted from B8 cells by acidic elution. Cells were removed from culture dishes with calcium- and magnesium-free medium and incubated at a density of 10^6 /ml for 1 h on ice in PBS containing the peptides in concentrations as described above. After 1 h, cells were resuspended in serum-free medium containing the peptides and left for 2 h at 37°C at a density of 2×10^5 /ml. Staining of the surface level of MHC class I molecules was performed as described above.

Cytolytic Assays. Target cells for cytolysis were labeled for 90 min with $\text{Na}_2^{51}\text{CrO}_4$. A standard 4-h cytolysis assay was performed in triplicate with 1,000 target cells and the indicated numbers of effector cells in two- or fourfold dilution steps as detailed in reference 26. All experiments were performed three times in triplicate cultures with two clones of each transfectant, except for the LMP2 T1A transfectant where four clones were analyzed.

Results and Discussion

Efficient Incorporation of the LMP2 T1A Subunit into the 20S Proteasomes. To investigate the consequence of a de-

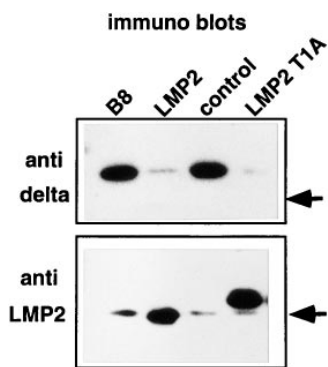


Figure 1. Expression of either LMP2 or the mutant LMP2 T1A results in complete replacement of subunit δ in 20S proteasomes. 20S proteasomes purified from the cell line B8 and from LMP2-, control-, and LMP2 T1A-transfected cells were examined for the presence of subunit δ (top) and LMP2 (bottom) by Western blotting. Even after strong overexposure of the immunoblots, only negligible amounts of residual δ subunit can be identified in 20S proteasomes of B8-LMP2 and B8-LMP2 T1A cells. Arrows, the position of a 21-kD marker protein.

finer active site elimination in the mouse 20S proteasome complex, the murine fibroblast cell line B8 was stably transfected with cDNAs either encoding a wild-type LMP2 or a mutated LMP2 subunit in which the active site threonine 1 residue was exchanged against alanine by site-directed mutagenesis (21). Overexpression of the LMP2 or LMP2 T1A subunits results in efficient incorporation of these subunits into the 20S proteasome complex (Fig. 1). The incorporation of the LMP2 proteins is associated with an almost complete exchange against subunit δ . Accordingly, by immunoblotting with anti- δ antibody only after overexposure of the enhanced chemiluminescence blot, negligible amounts of residual δ subunit could be identified in 20S proteasomes of B8-LMP2 and B8-LMP2 T1A cells. The slower electrophoretic mobility of LMP2 T1A also demonstrates that the NH₂-terminal prosequence is only partially cleaved, resulting in an NH₂-terminal extension of the subunit. Thus, overexpression of both the functional LMP2 subunit and the LMP2 T1A active site mutant subunit and the concomitant elimination of the active site bearing δ subunit from the 20S proteasome complexes allows production of a B8 cell line whose proteasome population possesses only four, instead of six, active sites. Interestingly, the functional elimination of this active site had no obvious phenotypic effect on the B8 fibroblast cells and appeared not to affect their growth rate.

Active Site Mutation Influences the Proteolytic Activity of 20S Proteasomes In Vitro. The effect of the active site mutation on the proteolytic activities of the 20S proteasome was tested by analyzing the peptide-hydrolyzing activities of 20S proteasomes from B8, B8-LMP2, and B8-LMP2 T1A with short fluorogenic peptide substrates (Table 1). Independent of the substrates used, neither the substitution of subunit δ by LMP2 nor the elimination of this active site by incorporation of LMP2 T1A has a significant effect on the V_{\max} of the 20S proteasome. This holds true for the trypsin-like activity monitored with the substrate Bz-Val-Gly-Arg-MCA as well as for the chymotrypsin-like activity measured with Z-Gly-Leu-Leu-MCA and Suc-Leu-Leu-Val-Tyr-MCA. Only in using MeOsuc-Gly-Leu-Phe-MCA was a reduction in V_{\max} by a factor of 2.9 measured

Table 1. Effects of Transfection of Wild-type B8 Cells with LMP2 and LMP2 T1A cDNAs on the Peptidase Activities of Proteasomes

Substrate	B8		B8-LMP2		B8-LMP2 T1A	
	V_{\max}	K_m	V_{\max}	K_m	V_{\max}	K_m
	$\mu\text{mol/h mg}$	μM	$\mu\text{mol/h mg}$	μM	$\mu\text{mol/h mg}$	μM
VGR-MCA	0.31	57	0.31	76	0.35	100
GGL-MCA	0.85	25	0.79	33	0.75	52
LLVY-MCA	1.11	29	1.20	41	1.33	70
GLF-MCA	0.18	35	0.14	35	0.05	83

V_{\max} and K_m values were determined with 20S proteasomes and the substrates Bz-Val-Gly-Arg-AMC for the trypsin-like activity, Z-Gly-Gly-Leu-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and MeOsuc-Gly-Leu-Phe-MCA for the chymotrypsin-like activity. The peptidyl-glutamyl-peptide hydrolyzing activity measured with the substrate Cbz-Leu-Leu-Glu-bNa was undetectable in both B8-LMP2 and B8-LMP2 T1A transfectants and is therefore not shown.

in the B8-LMP2 T1A mutant. Also, the K_m value, the measure for the binding affinity of substrates, was only moderately influenced by subunit substitution or active site mutation. For all substrates, we monitored an approximately twofold increase in the K_m for the LMP2 T1A proteasome. One possible reason for the observed increase in K_m values in the LMP2 T1A mutant could be that the NH₂-terminal extension of 8–10 amino acids of the mutant subunit influences the accessibility of the other active sites and hence the substrate binding affinity. Apart from this, the data suggest that the active site under investigation has little effect upon the trypsin and chymotrypsin-like peptide substrates, which is in agreement with the previous finding that the δ /LMP2 site affects the peptidyl glutamyl peptide-hydrolyzing activity (PDGH activity) of the proteasome complex. This activity is completely eliminated in these cells (data not shown). On the other hand, these data show that the different hydrolyzing activities, as monitored with unphysiologically short substrates, are in fact overlapping and that the attractive model of three different proteolytic specificities each mediated by one of three pairs of active sites is perhaps too simple. In a recent investigation, Eleuteri and coworkers (27) came to a similar conclusion by showing that short peptide hydrolyzing activities are overlapping and that different active sites cleave more than one type of short fluorogenic substrate. Interestingly, the incorporation of the LMP2 subunit into 20S proteasomes as such and not its activity seems to affect the enzymatic characteristics of the active sites of the neighboring subunit Z (β_2) and the more distant subunit MB1(β_5). This suggests once more (14) that the incorporation of this IFN- γ -inducible subunit may also influence the structure function relationship within the proteasome complex.

Active Site Deletion Leads to Reduced Surface Levels of MHC Class I Alleles. To investigate the effect of the δ /LMP2 active site mutation on the generation of antigenic peptides

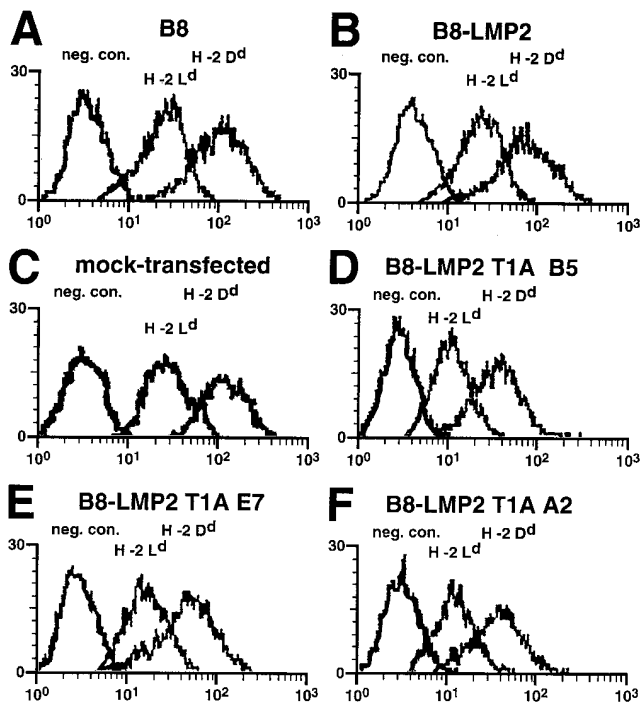


Figure 2. Active site mutation results in decreased surface levels of H-2L^d and H-2D^d alleles. Analysis of surface expression of the MHC class I molecules H-2L^d and H-2D^d by flow cytometry. Logarithmic fluorescence is plotted versus cell count. *neg. con.*, the second stage control only. Shown are the histograms of the stainings of B8 (A), of the LMP2 transfectant (B), and of B8 transfected with control vector (C). Also, several different clones of the LMP2 T1A transfectants were tested; the presented data of the clones LMP2 T1A B5 (D), LMP2 T1A E7 (E), and LMP2 T1A A2 (F) show a 40% reduction of H-2L^d and H-2D^d expression. Identical results were obtained in all independent experiments performed with two different anti-H-2L^d and anti-H-2D^d antibodies.

in vivo, we analyzed the cell surface expression of MHC class I molecules whose assembly and efficient transport to the cell surface is dependent on the loading with suitable peptides. Flow cytometric analysis of several independent B8-LMP2 T1A cell clones with allele-specific antibodies revealed an ~40% reduction in the cell surface expression of the H-2D^d and H-2L^d molecules when compared with B8, B-LMP2, or B8 mock-transfected control cells (Fig. 2). No difference in cell surface expression was found for the H-2K^d molecules (data not shown). That this is not a clonal effect is demonstrated by the finding that identical data were obtained with different B8-LMP2 T1A cell lines. These results suggest that the elimination of the two active sites restricts the overall quality of peptide generated, thus possibly limiting the supply of peptide and, in consequence, negatively affecting MHC class I molecule assembly and cell surface expression.

To test this hypothesis we took advantage of the observation that MHC class I molecules can reach the cell's surface without prior peptide binding when cells are incubated at 27°C and that empty MHC class I molecules can be stabilized by binding of externally added peptides (24, 25). B8-LMP2 T1A cells were therefore loaded either with a synthetic 9-mer peptide that binds to H-2L^d or peptides

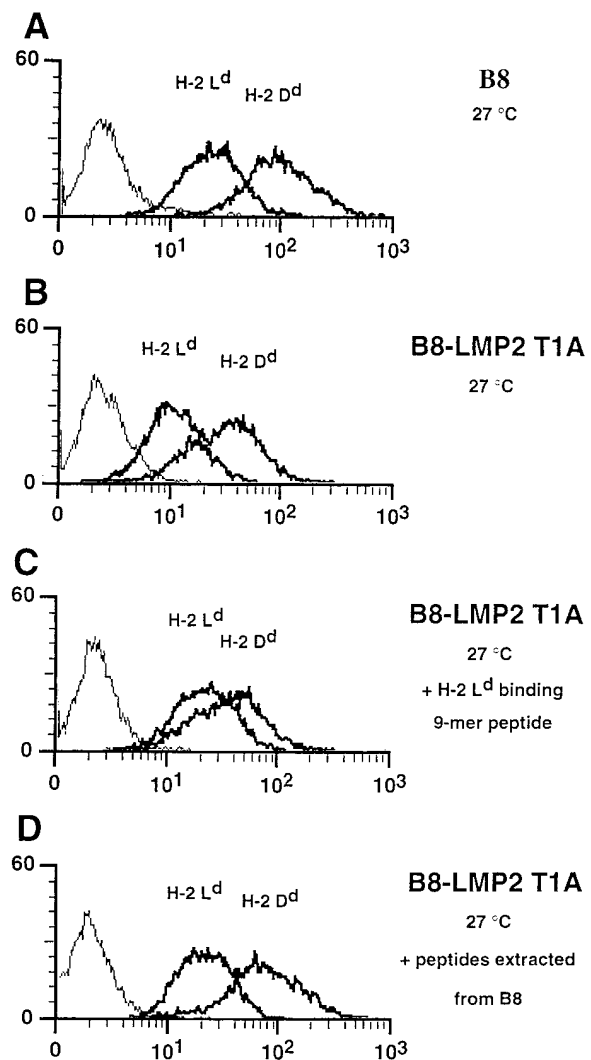


Figure 3. Reconstitution of surface expression of MHC class I on B8-LMP2 T1A cells by externally added peptides. Analysis of surface expression of the MHC class I molecules H-2L^d and H-2D^d by flow cytometry. Logarithmic fluorescence is plotted versus cell count. Curves drawn by thin lines represent the second stage control. Cells were cultured 18 h at 27°C in the presence of acid extracted peptides or a synthetic 9-mer known to bind H-2L^d. (A) Histograms of B8 cells (control) incubation with peptides does not alter MHC expression. (B) LMP2 T1A-transfected cells incubated in the absence of external peptides. (C) B8-LMP2 T1A cells incubated in the presence of a synthetic 9-mer known to bind to H-2L^d molecules. H-2L^d expression increases, whereas H-2D^d is not affected. (D) The externally added peptides from B8 cells restore the surface expression of MHC class I molecules on B8-LMP2 T1A cells.

extracted from B8 cells. As expected from its binding characteristics, the synthetic 9-mer peptide restores the level of H-2L^d, but not that of H-2D^d on B8-LMP2 T1A cells (Fig. 3 C). Furthermore, peptides extracted from nontransfected B8 cells were able to stabilize the levels of both MHC alleles on the surface of B8-LMP2 T1A cells (Fig. 3 D). These data demonstrate that it is indeed the lack of peptides that is responsible for reduced MHC class I expression on the surface of B8-LMP2 T1A cells. In support of this, pulse chase experiments and immunoprecipitation of H-2D^d and

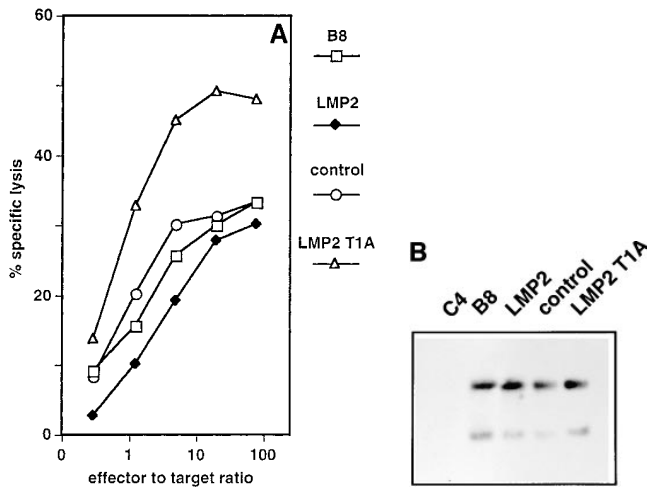


Figure 4. Active site mutation leads to enhanced presentation of the pp89 MCMV 9-mer peptide. (A) Analysis of MCMV pp89 antigen presentation in the cell lines B8 (white squares), LMP2 (black diamonds), control (white circles), and LMP2 T1A (white triangles). The susceptibility to lysis by MCMV pp89 (amino acids 168–176/H-2L^d)-specific cytotoxic T cells was determined in a 4-h chromium release assay. Effector/target ratios are plotted versus the percent of specific lysis. Values represent the means of three replicate cultures. Identical results were obtained in four independent experiments with two different B8-LMP2 clones and three different B8-LMP2 T1A clones. (B) Northern blot analysis of mRNA expression of the MCMV IE-1 protein pp89. C4 is a BALB/c mouse-derived fibroblast cell line from which B8 was obtained by transfection with the pp89 cDNA. B8 cells were stably transfected with the LMP2 cDNA expression construct (LMP2), vector only (control), or with a construct expressing the mutated cDNA of LMP2 (LMP2 T1A). Blotted poly A⁺ mRNA prepared from C4, B8, B8-LMP2, control, and B8-LMP2 T1A was hybridized with a pp89 cDNA probe. All cell lines contain almost identical amounts of pp89 mRNA according to PhosphorImager analysis.

H-2L^d molecules showed that these molecules are equally well expressed in all cell lines analyzed (data not shown). It may be argued that reduced temperatures can increase MHC expression independent of peptide supply. However, under the same experimental conditions, the number of K^d molecules does not increase at the cell surface at 27°C, even when peptides extracted from B8 cells are externally loaded. The elimination of active sites in the 20S proteasome complex therefore decreases the general amount of peptides available for binding to MHC class I H-2D^d and H-2L^d molecules. In contrast, the level of H-2K^d molecules is not reduced. Interestingly, all three haplotypes possess similar preferences for the COOH-terminal anchor residue but differ with regard to the residue preference at position 2 of the epitope. This indicates that the functional importance of the activity of the δ /LMP2 varies depending on the type of peptide products that have to be generated

for binding to a given MHC class I haplotype. In addition, despite the fact that the peptide hydrolyzing activities of the different active sites of the 20S proteasome are overlapping as deduced from in vitro data obtained with short fluorogenic peptide substrates (27), the δ /LMP2 active sites exert a specific cleavage property that is responsible for the in vivo generation of a specific peptide quality from natural protein substrates.

Enhanced Specific Cytolysis of Cells Expressing the LMP2 T1A Protein. So far the experiments showed that the functional elimination of two defined active sites in the 20S proteasome complex affects the quality of peptide generation and results in the downregulation of certain, but not all, MHC class I molecules. To determine how far the active site mutation influences the presentation of a specific peptide, we analyzed the different transfectant B8 cell lines with regard to their ability to present an immunodominant 9-mer peptide of the murine CMV (MCMV) pp89 to a H-2L^d-specific T cell line in a cytotoxicity assay. As shown in Fig. 4, B8 cells and B8 control-transfectant cells were lysed to the same extent, whereas B8-LMP2 cells were slightly less susceptible to lysis. In contrast, three- to five-fold less pp89/H-2L^d-specific cytotoxic T cells were required to lyse the B8-LMP2 T1A cells.

To exclude the possibility that increased pp89 expression in B8-LMP2 T1A cells was responsible for the observed effect, we investigated the expression of pp89 by Northern blot analysis since pp89 is quite stable at the posttranslational level (22). As shown in Fig. 4 B, no significant differences in the expression of pp89 can be detected in the investigated cell lines. Thus, despite the elimination of two active sites, sufficient pp89 antigen is generated to allow an increase in peptide-specific MHC class I presentation. Although in vitro experiments do not necessarily reflect the in vivo situation, it is interesting to note that in vitro digestions experiments of the pp89 25-mer synthetic polypeptide harboring the 9-mer epitope (15) show that the improved MHC class I presentation may be due to altered proteasomal processing properties. Although δ /LMP2 proteasomes have the tendency to destroy the epitope, mutant LMP2 T1A proteasomes do not use the internal cleavage site and thus seem to preserve the epitope (Ruppert, T., unpublished observations). In consequence, the increased maximum of lysis observed may be due to an increase in specific peptide supply. Considering that the overall H-2L^d surface expression is reduced in B8-LMP2 T1A cells, these experiments represent the first example that the specific elimination of a proteasomal active site, in this case δ /LMP2, may be beneficial for presentation of certain class I epitopes, despite reduced MHC class I expression.

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