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Altered Peptidase and Viral-Specific T Cell Response in *LMP2* Mutant Mice

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Summary

MHC class I molecules present peptides generated by processing of endogenously synthesized proteins to CD8⁺ T lymphocytes. Recently, large proteolytic complexes, termed proteasomes, were implicated in antigen processing. Two proteasomal subunits, LMP2 and LMP7, are encoded within the MHC class II region, but their precise role in antigen processing is unknown. We have generated mice that harbor a disruption in their *LMP2* gene. Proteasomes purified from spleen and liver of these mutant mice exhibit altered peptidase activities, and antigen-presenting cells showed reduced capacity to stimulate a T cell hybridoma specific for H-2D^b plus a nucleoprotein epitope of an influenza A virus. The mutant mice have reduced (60%–70% of wild type) levels of CD8⁺ T lymphocytes and generate 5- to 6-fold fewer influenza nucleoprotein-specific cytotoxic T lymphocyte precursors. These findings indicate that LMP2 influences antigen processing.

Introduction

Major histocompatibility complex (MHC) class I molecules present peptides, usually 8–10 aa in length, to CD8⁺ cytotoxic T lymphocytes (CTL) (reviewed by Townsend and Bodmer, 1989; Germain and Margulies, 1993). Most antigenic peptides are derived by processing of proteins in the cytosol. These peptides are subsequently translocated to the lumen of the endoplasmic reticulum (ER) by a heterodimeric translocator, termed the transporter associated with antigen presentation (TAP). Upon their entry into the ER, peptides assemble with newly synthesized class I heavy chains and β2-microglobulin, and this trimolecular complex is then transported to the cell surface for presentation to CD8⁺ T lymphocytes.

Current knowledge of the proteolytic machinery responsible for the generation of class I MHC-binding peptides is limited. Recent studies suggest a role for large proteolytic enzyme complexes, termed proteasomes. Proteasomes consist of 13–15 distinct subunits and contain multiple active sites that catalyze peptide bond cleavage on the carboxyl side of hydrophobic, basic, and acidic amino acid residues (Goldberg, 1992; Goldberg and Rock, 1992; Rivett, 1993). They constitute the catalytic core of a larger complex involved in the adenosine triphosphate (ATP)-dependent degradation of proteins targeted for destruction by conjugation with ubiquitin (Goldberg and Rock, 1992; Hershko and Ciechanover, 1992). Proteasomes possess proteolytic specificities that are appropriate for cleaving antigenic proteins to class I MHC-binding peptides: purified proteasome fractions were capable of degrading two full-length proteins, ovalbumin (OVA), and β-galactosidase in vitro to produce the peptide epitopes that are found naturally in association with class I MHC molecules (Dick et al., 1994). A role for the ubiquitin-dependent degradation pathway in antigen presentation has been identified. Modification of the amino terminus of a viral protein with large charged amino acids, to promote degradation by this pathway, enhances presentation by class I MHC molecules (Townsend et al., 1988). Similarly, cells with a defect in ubiquitination have reduced capacity to present cytoplasmic OVA antigens to CD8⁺ T cells (Michalek et al., 1993). Furthermore, experiments with proteasome-specific inhibitors have demonstrated that the proteasome is the major pathway for generating class I–presented peptides (Rock et al., 1994).

Two subunits of the proteasome, termed low molecular weight protein (LMP) 2 and LMP7, are encoded within the MHC class II locus, in close proximity to the genes (*TAP1* and *TAP2*) that encode the subunits of the peptide transporter (Martinez and Monaco, 1991; Brown et al., 1991; Glynne et al., 1991; Kelly et al., 1991; Ortiz-Navarrete et al., 1991). LMP2 and LMP7 associate with the proteasome and are legitimate members of the proteasome gene family, since they have sequence similarities with other subunits (Glynne et al., 1991; Kelly et al., 1991). LMP2 and LMP7 are inducible by interferon-γ (IFNγ) (Yang et al., 1992, Brown et al., 1993) and exhibit amino acid sequence polymorphisms (Monaco and McDevitt, 1982; Kelly et al., 1991; Ortiz-Navarrete et al., 1991; Zhou et al., 1993), features shared with the class I MHC molecules and the TAPs.

Incorporation of the LMP2 and LMP7 subunits into proteasomes alters the cleavage specificity of the complexes (Driscoll et al., 1993; Gaczynska et al., 1993; Aki et al., 1994; Boes et al., 1994). Proteasomes isolated from hu-

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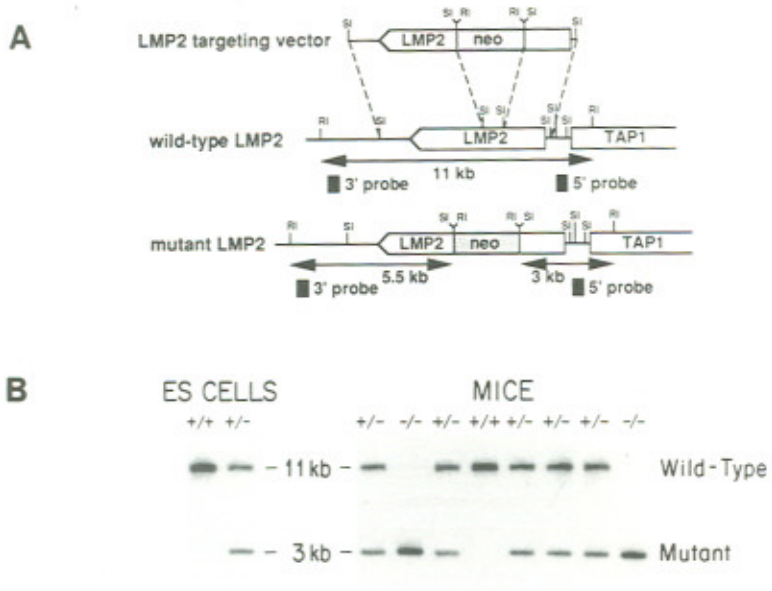

Figure 1. Generation of *LMP2* Mutant Mice

(A) The *LMP2* locus and targeting construct. The *LMP2* and *TAP1* genes are shown. An 800 bp fragment of the *LMP2* gene was replaced with a *neo* gene. *LMP2* 5′ and 3′ flanking probes used for screening of ES cell clones and mice are indicated, together with the expected size of hybridizing restriction fragments in wild-type and mutant *LMP2* alleles. Restriction site abbreviations: RI, EcoRI; SI, SmaI.

(B) Southern blot analysis of representative ES cell clones and tail biopsies. Genomic DNA was isolated from an ES cell clone with a mutant *LMP2* allele and wild-type control (left) and from a litter of eight mice from a heterozygote intercross (right). DNA was digested with EcoRI and hybridized with the 5′ flanking probe (see A). Wild-type and targeted alleles are indicated. Two mice in the litter are homozygous for the mutation.

man lymphoblastoid cells (.174 or T2 cells) with a large deletion in the MHC that encompasses both *LMP2* and *LMP7* exhibit a reduced capacity to cleave peptides after basic and hydrophobic residues (Driscoll et al., 1993; Gaczynska et al., 1993) and an enhanced capacity to cleave after acidic residues (Gaczynska et al., 1993). The opposite effect was seen with proteasomes isolated from cells that were treated with IFNγ to induce LMP expression (Gaczynska et al., 1993; Aki et al., 1994). Since most peptides that actually bind class I MHC molecules terminate in hydrophobic or basic residues (Falk and Rötzschke, 1993), it is expected that incorporation of LMPs in proteasomes favors the production of peptide species that can assemble with MHC class I molecules.

A number of observations have cast doubt on the proposed role of LMPs in antigen processing. First, the human mutant cell lines that lack LMP2 and LMP7, but with a peptide transporter reconstituted by transfection of the genes that encode the individual transporter subunits, express normal levels of class I MHC at the cell surface (Arnold et al., 1992; Momburg et al., 1992), suggesting that lack of LMP2 and LMP7 does not limit the supply of peptides to class I MHC molecules. Second, these mutant cells were also capable of presenting antigens to CTL, and (for most antigens tested) presentation was as efficient as with wild-type cells (Arnold et al., 1992; Yewdell et al., 1994; Zhou et al., 1994). This has led to the suggestion that LMP2 and LMP7 are not required for efficient antigen presentation, and that these proteins may have a more specialized role in the production of class I MHC-associated peptides.

The characteristics of antigen presentation, T cell development, and T cell responsiveness are analyzed here for mice with a homozygous disruption in the *LMP2* gene. Contrary to previous reports from experiments performed only in cell culture systems, significant effects were observed.

Results

Generation of *LMP2* Mutant Mice

To generate *LMP2*-deficient mice, a 800 bp region from the *LMP2* gene, encompassing part of the second exon and part of the second intron, was replaced with a neomycin resistance (*neo*) gene in embryonic stem (ES) cells (Figure 1). ES cell clones with the desired homologous recombination were injected into mouse blastocysts, which were subsequently implanted into foster mothers to generate chimeric offspring. Chimeric mice were mated with C57BL/6 mice to obtain heterozygous mutant mice, which were intercrossed to homozygozity. Insertion of the *neo* gene near the *TAP1* gene (see Figure 1A) did not have a detectable effect on *TAP1* RNA expression (data not shown).

The mutant animals were viable, appeared healthy, had no gross anatomical abnormalities, and lived to at least 1 year of age.

Mutant Mice Lack the LMP2 Protein

Cell extracts from metabolically labeled mutant and wild-type cells were immunoprecipitated with a rabbit anti-rat proteasome serum to demonstrate that the LMP2 subunit is absent from proteasomes of mutant mice. Immunoprecipitates were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Figure 2). This also showed that incorporation of LMP7 in proteasomes was not significantly affected by the absence of LMP2. The loss of LMP2 was also associated with differences in the relative intensities of some other proteasomal subunits (Figure 2), indicating that absence of LMP2 affects the overall composition of the proteasome.

Western blotting with specific antibodies against LMPs was used to examine LMP2 and LMP7 expression in partially purified proteasomes from mutant and wild-type mice. No LMP2 was found in the proteasomes isolated

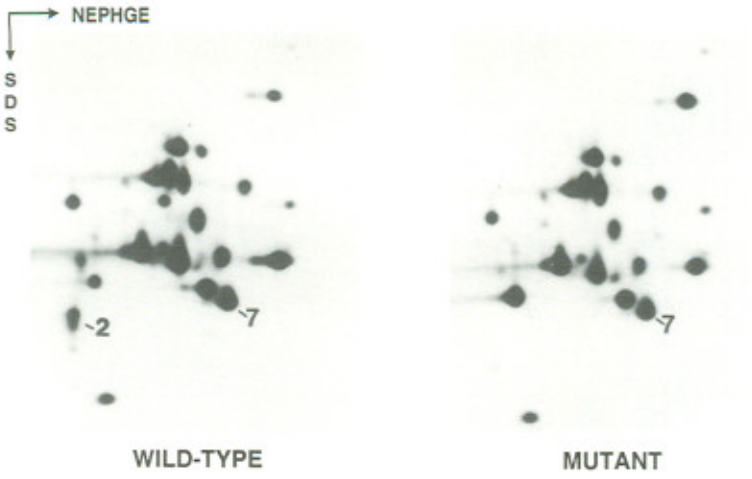

Figure 2. *LMP2* Mutant Mice Lack the LMP2 Protein

Concanavalin A–induced lymphoblasts from *LMP2* (+/+) and *LMP2* (−/−) mice were metabolically labeled with [^{35}S]methionine and detergent extracts from these cells were immunoprecipitated with a rabbit anti-rat proteasome serum. Samples were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), with nonequilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension and SDS–PAGE in the second dimension. The positions of LMP2 and LMP7 subunits are indicated. Note that in addition to the loss of LMP2, differences in the intensities of some other proteasomal subunits can be observed.

from the mutants, and the proteasome content and LMP7 levels in tissues from mutant and wild-type mice were indistinguishable. The amounts of LMP2 were variable in different tissues from control animals. Proteasomes isolated from the spleen contained the highest amounts of LMP2. Levels in the liver were 24 ± 1% of those in spleen (mean ± SEM; n = 4), but very low amounts were detected in muscle (2 ± 1%, n = 3) and brain (<1%, n = 3). Spleen proteasomes also contained the most LMP7, with values for the liver 45 ± 4% of that in spleen and very low in muscle (5 ± 1%) and brain (3 ± 1%). In general, the LMP2 and LMP7 profiles correlate with the organ distribution of MHC class I glycoprotein expression in the absence of IFNγ treatment (Chamberlain et al., 1991).

Altered Peptidase Activities

Proteasomes have distinct sites that cleave small peptides on the carboxyl side of hydrophobic, basic, and acidic amino acids (Goldberg, 1992; Goldberg and Rock, 1992). Three fluorogenic substrates were used to determine whether lack of *LMP2* expression influences this peptidase activity. Proteasomes isolated from mutant spleen and liver degraded hydrophobic substrate (Suc-LLVY-MCA) and basic substrate (Boc-LRR-MCA) at significantly lower rates than those from wild-type mice (Table 1). This difference was larger for spleen than for liver (Table 1; Figure 3), reflecting the greater divergence in LMP2 content. In contrast, degradation of the acidic substrate, Cbz-LLE-βNA, was almost 2-fold higher for mutant than for wild-type spleen proteasome (Table 1; Figure 3). No significant differences in proteasomal peptidase activities were observed for proteasomes from muscle or brain that lack the LMP subunits (Table 1). The degradation rates of [^{14}C]casein did not differ significantly between wild-type and mutant preparations from any tissue (data not shown), indicating that the observed changes were not a consequence of variability in the total amount of proteolytic activity in the proteasome fractions. Thus, the divergence in the peptidase activity for proteasomes isolated from various tissues of normal and mutant mice is correlated with LMP2 content; constitutive *LMP2* expression is highest in the spleen and lowest in muscle and brain.

No Dramatic Effect on Class I MHC Cell Surface Expression

Nearly all class I peptides presented to the immune system harbor hydrophobic or basic residues at their carboxyl terminus (Falk and Rötzschke, 1993). Consequently, the enhanced cleavage of peptides after hydrophobic and basic residues resulting from incorporation of the LMP2 subunit should favor the production of the types of peptides found on MHC class I molecules. Since the efficient assembly and transport of class I molecules to the cell surface require peptides (Townsend et al., 1989, 1990; Ljunggren et al., 1990; Schumacher et al., 1990; Van Kaer et al., 1992), we examined the pattern of surface class I MHC expression of cells from mutant mice by FACS analysis. Staining intensity of mutant and wild-type cells with anti-H-2D^b and anti-H-2K^b antibodies was comparable (Figure 4).

Reduced Capacity to Present an Influenza Virus Antigen to T Cells

The normal expression of MHC class I implies that the supply of peptides to class I molecules is not limiting, but this finding does not indicate whether the loss of *LMP2* has altered the characteristics of the peptides that are presented. The capacity of cells from mutant and wild-type mice to process and present antigen to CD8$^+$ T cells following infection with an influenza A virus was thus assayed with a T cell hybridoma (RF36.84) specific for the influenza nucleoprotein (NP) peptide 366–374 (IF-NP$_{366-374}$) plus H-2D^b. Virus-infected lipopolysaccharide (LPS)-induced splenic lymphoblasts and peritoneal exudate cells (PEC) from mutant mice were less efficient stimulators than comparable control cells (Figures 5A and 5C). However, mutant and wild-type cells were equally well sensitized for recognition by the hybridoma when exogenous IF-NP$_{366-374}$ peptide was added to the culture (Figures 5B and 5D), establishing that the defect is in generating peptides rather than presentation.

We also tested the capacity of mutant cells to process OVA antigens for presentation to an H-2K^b-restricted OVA$_{257-264}$-specific CTL clone (4G3), and an OVA$_{257-264}$-specific T cell hybridoma (RF33.70). No significant differences were observed (data not shown; three experiments

Table 1. Rates of Peptide Degradation by Proteasomes from Various Tissues of Control and Mutant Mice

	Hydrophobic substrate			Basic Substrate			Acidic Substrate		
	Control	Mutant		Control	Mutant		Control	Mutant	
Tissue	V_{max} (μmol/mg/h)		(percentage of change)	V_{max} (μmol/mg/h)		(percentage of change)	V_{max} (μmol/mg/h)		(percentage of change)
Spleen n = 6	0.31 ± 0.01	0.14 ± 0.03	(−57)*	0.28 ± 0.02	0.15 ± 0.04	(−49)*	0.23 ± 0.01	0.39 ± 0.05	(+70)#
Liver n = 4	0.96 ± 0.07	0.66 ± 0.03	(−24)*	0.47 ± 0.01	0.32 ± 0.03	(−31)#	0.76 ± 0.04	0.74 ± 0.09	(−5)ns
Muscle n = 3	0.12 ± 0.01	0.10 ± 0.01	(−20)ns	0.16 ± 0.02	0.13 ± 0.01	(−20)ns	0.18 ± 0.03	0.19 ± 0.04	(+9)ns
Brain n = 3	0.18 ± 0.02	0.17 ± 0.03	(−15)ns	0.19 ± 0.02	0.16 ± 0.01	(−15)ns	0.17 ± 0.03	0.16 ± 0.03	(−5)#

Mean ± SEM; *$p < 0.001$; *$p < 0.01$; #$p < 0.05$; ns, not significant (t test).
For each experiment; tissues were obtained from 3–5 mice and homogenized.

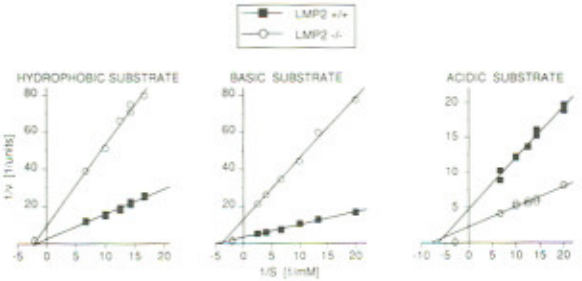

Figure 3. Effects of *LMP2* Mutation on Peptidase Activities of Proteasomes from Murine Spleen

Data shown are typical rates for hydrolysis of fluorogenic substrates (various concentrations; S) by proteasomes isolated from five spleens of control and mutant mice. V_{max} values for the hydrophobic substrate, Suc-LLVY-MCA, 0.39 U (each unit equals 1 μmol/mg/hr) for control and 0.11 U for mutant; for basic Boc-LRR-MCA, 0.29 U for control and 0.08 U for mutant; for acidic Cbz-LLE-βNA, 0.22 U for control and 0.44 U for mutant. K^M values for Suc-LLVY-MCA, 0.53 mM for control and 0.46 mM for mutant; for Boc-LRR-MCA, 0.20 mM for control and 0.25 mM for mutant; for Cbz-LLE-βNA, 0.16 mM for control and 0.12 mM for mutant.

for the CTL clone each with concanavalin A blasts from two mutant and two wild-type mice with varying amounts of OVA; two experiments for the hybridoma each with LPS blasts from two mutant and two wild-type mice and a fixed amount of OVA).

Reduced Levels of CD4⁻CD8⁺ T Cells

Class I–peptide complexes are essential for the intrathymic differentiation of $CD4^-CD8^+$ T lymphocytes (Rothenberg, 1992; Ashton-Rickardt et al., 1993). Lymphoid organs from mutant and wild-type mice were thus compared for the prevalence of various T cell subsets. The numbers of $CD4^-CD8^+$ T cells were reduced 36% in blood, 22% in spleen, and 49% in thymus (Figure 6). The spectra of T cell receptor Vβ chains of $CD4^-CD8^+$ T cells were identical (data not shown), establishing that the loss of *LMP2* has no obvious effect on the T cell repertoire. Neither the $CD4^+CD8^-$ T cells in spleen, blood, and thymus nor the $CD4^+CD8^+$ thymocytes showed any change in numbers (data not shown).

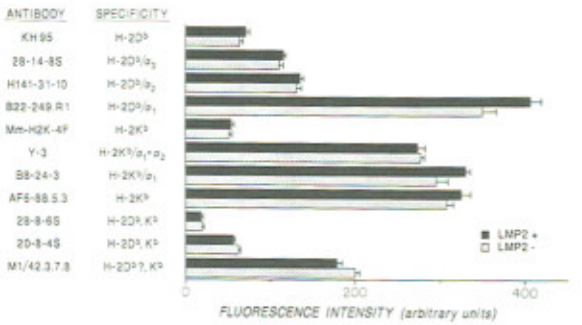

Figure 4. Class I MHC Cell Surface Expression on Spleen Cells from Mutant Mice

Spleen cells from *LMP2* (+/+) and (−/−) mice were stained with the indicated anti-class I antibodies and analyzed by FACS. Mean ± SEM of 9–10 replicate measurements from individual mice are shown.

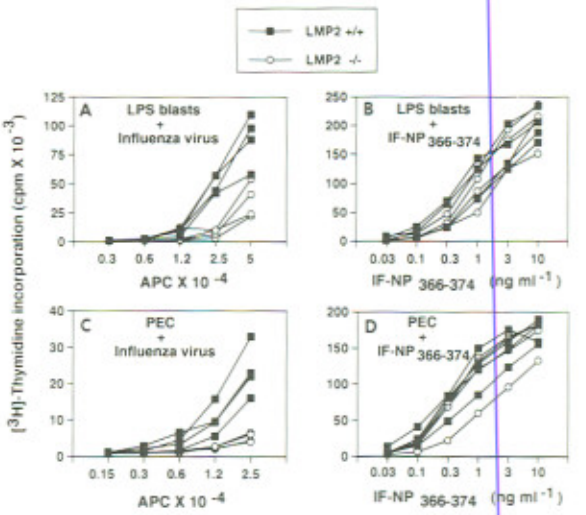

Figure 5. Cells from Mutant Mice Are Less Efficient in the Presentation of Influenza Virus Antigens to a IF-$NP_{366-374}$-Specific T Cell Hybridoma

As APCs, LPS-induced splenic lymphoblasts (A, B) or PEC (C, D) were used. APCs were infected with influenza virus (A, C) and the indicated numbers (per well) were mixed with cultures of the T–T hybridoma RF36.84. Stimulation of the hybridoma by IF-$NP_{366-374}$ plus D^b complexes was measured with an interleukin-2 (IL-2) assay. As controls, uninfected APCs (10^5 per well) were treated with serial 3-fold dilutions of synthetic IF-$NP_{366-374}$ peptide and then tested for stimulation of the hybridoma. Results for individual mice (four mutant and four wild-type) are shown. For the highest number of APCs tested, there was a 2.5-fold difference ($p < 0.01$) between mutants and controls in case of LPS blasts and a 4.7-fold difference ($p < 0.01$) for PEC. Similar results were obtained in three other independently performed experiments; specifically, results for LPS blasts: experiment 1, 2.7-fold difference ($n = 4$; $p > 0.1$); experiment 2, 1.6 ($n = 4$; $p > 0.1$); experiment 3, 2.9 ($n = 4$; $0.05 < p < 0.1$); results for PEC: experiment 1, 1.4 ($n = 4$; $p > 0.1$); experiment 2, 4.7 ($n = 4$; $0.01 < p < 0.02$); experiment 3, 5.1 ($n = 4$; $0.05 < p < 0.1$).

Effects of *LMP2* Mutation on Viral-Specific T Cell Responses

CTLs play an important role in recovery from acute viral infections (Doherty et al., 1992). Mutant and wild-type animals were acutely infected with an influenza A virus or with a parainfluenza type I (Sendai) virus. The frequencies of virus-specific CTL precursors (CTLp) in the mediastinal lymph nodes were determined 1 or 2 weeks after infection. The response normally peaks after about 7 days, while analyzing again at day 14 allows time for compensatory effects to develop. Mutant mice infected with influenza virus consistently had 5- to 6-fold lower CTLp frequencies than infected control mice when T cells were assayed on peptide-pulsed MHC class I^+ II^- wild-type target cells (Figure 7). Since a similar reduction in CTLp frequency was evident when infectious virus was used to prepare the target cells (data not shown), the response in mutant mice was not redirected to a different epitope. Furthermore, the decrease in CTLp frequency was not a function of the slightly smaller numbers of $CD8^+$ T cells in the lymph nodes. No difference was detected in the CTLp frequency of wild-type and mutant mice infected with Sendai virus (Figure 7).

Figure 6. *LMP2* Mutant Mice Have Reduced Levels of $CD4^-CD8^+$ T Lymphocytes

Blood, spleen, and thymus cells from wild-type (+/+ or +/−) and mutant (−/−) mice were stained with anti-CD4 and anti-CD8 antibodies and analyzed by FACS. The percentage of $CD4^-CD8^+$ T cells among the total number of lymphocytes is presented. Results for individual mice are shown, along with statistical data (mean ± SEM and p values).

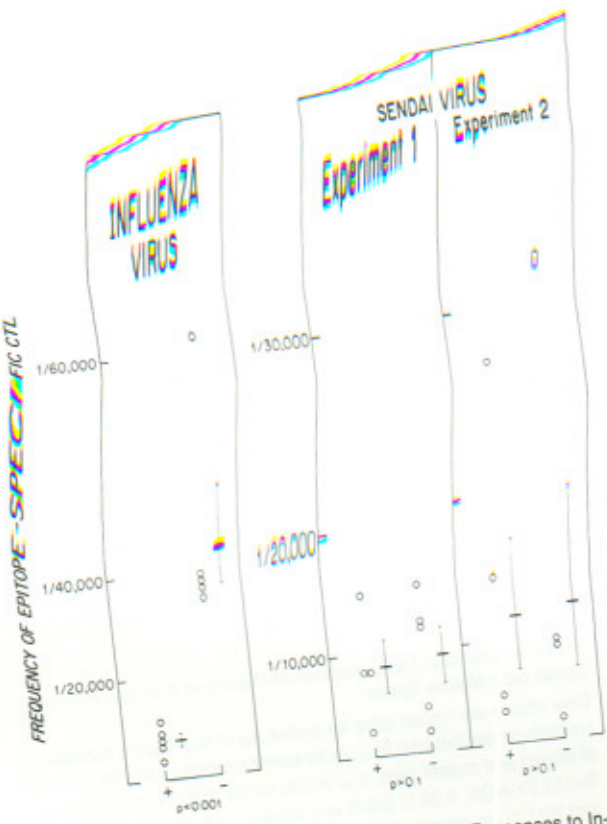

Figure 7. *LMP2* Mutant Mice Exhibit Reduced CTL Responses to Infection with Influenza Virus but not Sendai Virus

Mice were acutely infected with influenza or Sendai virus. After 1 or 2 weeks of infection, the CTLp specific for the immunodominant epitope was measured in the mediastinal lymph nodes. Results are presented as the frequency of epitope-specific CTL among all lymphocytes in mediastinal lymph nodes. Results for individual mice are shown, along with statistical data (mean ± SEM and p values). A second experiment using influenza-infected mutant and control mice gave similar results.

Discussion

The identification of the *LMP* genes within the MHC region first suggested a potential connection between the products of these genes and the immune system. The finding that the LMP subunits alter the activity of the proteasome in a manner that should favor the generation of antigenic peptides (Driscoll et al., 1993; Gaczynska et al., 1993; Aki et al., 1994) supported this view, but the evidence has been indirect. The present findings clearly strengthen the proposed role of the proteasome in the MHC class I pathway and provide the first direct evidence that LMP subunits can enhance presentation of some antigens.

Several lines of evidence indicate that the defect in the *LMP2* mutant mice is associated with antigen processing. First, the peptidase activities of proteasomes from mutant mice are altered. These changes resemble the pattern seen previously for proteasomes of the MHC deletion mutant cell line that lacks both *LMP2* and *LMP7* genes (Driscoll et al., 1993; Gaczynska et al., 1993), and are opposite to the effects seen following induction of the LMPs by IFNγ treatment (Gaczynska et al., 1993; Aki et al., 1994). Moreover, transfection of cells with *LMP2* cDNA enhances cleavage after basic residues and suppresses hydrolysis after acidic residues (Gaczynska et al., 1994). However, for reasons that are unclear, the hydrophobic activity that was reduced in the *LMP2* mutant mice did not change with *LMP2* transfection. These changes resulting from the absence of LMP2 would predict less efficient generation of class I MHC-binding peptides. Second, mutant mice generated decreased numbers of influenza virus–specific

of peptide in T cell positive selection indicate that the type of peptide bound to class I molecules influences the $CD4^-CD8^+$ T cell repertoire and that an increased complexity of the MHC-bound peptides enhances the repertoire of selected cells (Ashton-Rickardt et al., 1993; Hogquist et al., 1993). The reduced number of $CD4^-CD8^+$ T cells in the *LMP2* mutant mice may therefore reflect differences in the composition, complexity, or both of peptides bound to class I molecules on thymic epithelial cells, resulting from altered processing of self-proteins.

The defect seen in the influenza model is selective: mutant and wild-type mice were very similar in their capacity to present OVA to T cells, and the CTL response of mutant and wild-type mice to infection with Sendai virus was indistinguishable. This, together with the fact that mutant animals express normal levels of class I molecules, indicates that the generation of many endogenous peptides does not require LMP2. Perhaps some of these peptides are not generated by the proteasome, in which case the loss of LMP2 would not be expected to alter presentation in association with class I MHC glycoprotein. Alternatively, the proteasome might produce such peptides in an LMP2-independent manner. Even so, the influenza data indicate that LMP2 can be important for the generation of class I MHC-restricted T cell responses.

Proteasomes are responsible for the elimination of pro-

that the general housekeeping functions of proteasomes are not adversely affected by the absence of the LMP2 subunit. This suggests that the MHC-encoded subunits of the proteasome (LMP2 and LMP7) have a more specialized role than the subunits that are not encoded within the MHC, presumably in antigen processing.

The absence of LMP2 resulted in changes in the proteasomal subunit composition. Similar observations were made with the human MHC deletion mutant cell lines that lack both LMP2 and LMP7, and with cells where LMPs were induced by IFNγ (Yang et al., 1992; Brown et al., 1993; Aki et al., 1994; Boes et al., 1994; Früh et al., 1994). Taken together, these data suggest that LMP2 and LMP7, at least in part, control the recruitment of other subunits into the proteasome. Part of the phenotype of the *LMP2* mutant mice may therefore be only indirectly caused by the lack of LMP2, via changes induced in proteasome structure.

In conclusion, our data indicate that LMP2, and presumably also LMP7, induce alterations in the subunit organization of proteasomes, which in turn modify the proteolytic capabilities of these complexes in a way that is beneficial for presentation of MHC class I–restricted antigens to the immune system.

Experimental Procedures

Production of *LMP2*-Defective Mice

An *LMP2* targeting vector was constructed from C57BL/6 cosmid clones described before (Van Kaer et al., 1992). It was prepared in a quatrimolecular ligation reaction, using a 2.1 kb SmaI fragment from the 5′ end of *LMP2*, a 1.8 kb EcoRI–ClaI fragment containing a *neo* gene driven by the *PGK1* promoter (a gift from Dr. M. Rudnicki), a 3.4 kb SmaI fragment from the 3′ end of *LMP2*, and the plasmid pBluescript (Stratagene), digested with appropriate restriction enzymes. This construct was designed to delete 800 bp from the *LMP2* gene that encompasses a portion of exon 2 and intron 2. Strain 129–derived E14 ES cells (Hooper et al., 1987) were transfected with the targeting vector, and G418-resistant colonies were selected and isolated as described (Van Kaer et al., 1992). Genomic DNA from individual clones was digested with EcoRI and hybridized with probes from the 5′ (a 550 bp SmaI fragment) and 3′ (a 550 bp SphI–ClaI fragment) *LMP2*-flanking region (see Figure 1A). The wild-type *LMP2* allele gave an 11 kb fragment for both probes, the mutant allele gave a 5.5 kb fragment for the 5′ probe and a 3 kb fragment for the 3′ probe. Out of 288 clones analyzed, 5 carried the desired mutation. Of these, three were selected for injection into C57BL/6 blastocysts. Of the injected clones, two gave rise to chimeric offspring (agouti on black coat color). These were then mated with C57BL/6 mice to score for germline transmission. Chimeras (four male chimeras out of five and one female chimera out of three) from one clone resulted in agouti offspring. Germline transmission was confirmed by Southern blotting of tail DNA. Heterozygous mutant mice were intercrossed to homozygozity. All mice used in this study were F2 or F3 animals and were typed by Southern blotting of tail DNA. Breeding pairs of the mice were always housed in autoclaved cages with autoclaved food and water. Adult mutant mice were housed in normal cages, except for mice used in virus infections, which were maintained under pathogen-free conditions. In most experiments, strain 129 and C57BL/6 mice were assayed in parallel to exclude differences caused by the genetic background of these mice.

Immunoprecipitations

Concanavalin A (2.5 μg/ml)–induced splenic blast cells were metabolically labeled for 4 hr at 37°C with 500 μCi [^{35}S]methionine (1,200 Ci/mol, Amersham) per 2 × 10^7 cells. Cells were lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris–HCl [pH 7.5], 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride). Extracts were immunoprecipitated with a rabbit serum raised against rat proteasomes (obtained from Dr. J. Driscoll; described by Brown et al., 1991). Immunoprecipitates were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with nonequilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension and SDS–PAGE in the second dimension, as described (Celis and Bravo, 1984).

Proteasome Purification

Proteasomes were partially purified by differential centrifugation of tissue homogenates (Tanaka et al., 1986). In brief, organs from mice were first washed in homogenization buffer (50 mM Tris–HCl [pH 7.4], 5 mM $MgCl_2$, 1 mM dithiotreitol, 2 mM ATP, and 0.25 M sucrose) and then homogenized in a Dounce homogenizer. The resulting suspension was centrifuged at 10,000 × g for 20 min. The supernatant was collected and centrifuged at 100,000 × g for 1 hr. The supernatant was centrifuged again for 5 hr at 100,000 × g and the resulting protein pellet was dissolved in buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiotreitol, 2 mM ATP, and 20% (v/v) glycerol, and stored at −80°C.

Western Blotting

Western blots were performed according to standard procedures with antibodies specific for LMP2 or LMP7 (obtained from Dr. K. Tanaka; described by Aki et al., 1994; Gaczynska et al., 1994).

Peptide Hydrolysis Assay

Rates of cleavage (V, in nanomoles substrate cleaved per milligram protein per hour) by proteasome fractions were analyzed with different substrate concentration (S, in millimolars) in homogenization buffer without ATP and supplemented with 5 U/ml apyrase. Reactions were quenched with 1% SDS and fluorescence determined using an excitation of 380 nm and emission of 460 nm for MCA, and an excitation of 336 nm and emission of 415 nm for βNA. Substrates used were: Suc-LLVY-MCA (hydrophobic substrate), succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; Boc-LRR-MCA (basic substrate), N-tert-butoxycarbonyl-Leu-Arg-Arg-7-amido-4-methylcoumarin; Cbz-LLE-βNA (acidic substrate), benzoylocarbonyl-Leu-Leu-Glu-β-naphthylamide.

Flow Cytometric Analyses

The following class I monoclonal antibodies were used: fluorescein isothiocyanate–labeled KH95 (H-2D^b specific), 28-14-8S (D^b), H141-31-10 (D^b), biotin-labeled B22-249. R1 (D^b), Mm-H2K-4F (K^b), Y-3 (K^b), R phycoerythrin-labeled AF6-88.5.3 (K^b), 28-8-6S (D^b, K^b), 20-8-4S (D^b, K^b), and M1/42.3.7.8 (D^b?, K^b) (obtained from Pharmingen, the American Type Culture Collection, Dr. H. N. Eisen, or Dr. H. L. Ploegh). Other monoclonal antibodies were anti-CD4 (biotin-labeled), anti-CD4 (allophycocyanin-labeled), anti-CD8a (fluorescein isothiocyanate–labeled), anti-CD3ε (R phycoerythrin-labeled) (all from Pharmingen), and anti-Vβ-specific antibodies (Vβ2, Vβ3, Vβ5, Vβ6, Vβ8.1+8.2, Vβ8.3, Vβ9, Vβ10, Vβ11, Vβ13, Vβ14, and Vβ17) (obtained from Pharmingen, or from Dr. H. N. Eisen).

Cell suspensions were prepared according to standard procedures and incubated in staining buffer (phosphate-buffered saline, 1% bovine serum albumin, and 0.1% NaN_3) with monoclonal antibodies for 30 min at 4°C. Cells were then washed and, where appropriate, incubated with fluorescein isothiocyanate–labeled goat anti-mouse immunoglobulin G (IgG) (Southern Biotechnologies Associates, Incorporated) or streptavidin–R phycoerythrin (Pharmingen) for another 15 min. After washing, cells were analyzed using a FACScan flow cytometer (Becton-Dickinson). Dead cells were excluded from the analysis by staining with propidium iodide, or based on the forward and sideways light scattering properties.

In Vitro Antigen Presentation Assays

The RF36.84 (IF-$NP_{366–374}$ plus D^b specific) and RF33.70 ($OVA_{257–264}$ plus K^b specific) T–T hybridomas have been described (Rock et al., 1990a, 1991). Influenza virus infections were performed by incubation of antigen-presenting cells (APCs) (0.5–1 × 10^7/ml in OPTIMEM media) with or without influenza A 1968 (200 μl of allantoic fluid per ml; about 2 × 10^3 HAU/ml) for 3 hr at 37°C. Ovalbumin was introduced into the cytoplasm of APC by osmotic lysis of pinosomes (Moore et al., 1988). Cells were then washed and mixed with the RF36.84 or RF33.70 hybridoma. Culture supernatant (100 μl) was removed from

duplicate cultures and assayed for interleukin-2 content as described (Rock et al., 1990b).

The 4G3 CTL clone (obtained from Dr. H. N. Eisen) is specific for $OVA_{257-264}$ plus K^b (Walden and Eisen, 1990). [^{51}Cr] release assays were performed as described (Van Kaer et al., 1992).

Virus Infections and Determination of CTL Precursor Frequencies

In vivo virus infections and determination of CTLp frequencies were performed as described (Allan et al., 1990; Hou and Doherty, 1993). The A/HKx31 (H3N2) influenza A virus (a laboratory-generated recombinant between A/PR8/34 and A/Aichi) and the Enders strain of Sendai virus were used. Anesthetized mice were infected intranasally with 50 μl of phosphate-buffered saline containing 240 HAU of the A/HKx31 virus or 200 EID50 of the Sendai virus. T cell precursors were measured by limiting dilution analysis (LDA), as described (Lefkovitz et al., 1979; Hou and Doherty, 1993). C57BL/6 splenocytes pulsed with synthetic IF-$NP_{366-374}$ peptide (ASNENMETM) or SV-$NP_{324-332}$ peptide (FAPGNYPAL) were used as stimulators and MHC class I^+ II^- H-2^b MC57G cells (Bataillon et al., 1975) pulsed with the relevant peptide were used as targets.

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