H1 (0.25 mg/ml) (Boehringer Mannheim), and 10 μCi of [γ-^{32}P]ATP (Amersham). After incubation for 10 min at 30°C, the reaction mixture was briefly centrifuged, and 50 μl of SDS sample buffer were added to the supernatant, which was then boiled for 5 min. Proteins were separated by SDS-PAGE (12% gel), and ^{32}P-labeled histone H1 was visualized by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

21. RD 680 (*MATα ura3-52 leu2-3,112::GAL-STE4::LEU2 reg1-501 GAL$^+$ pep4-3 prd1-1122*) transformed with pTP20 (p*GAL-FAR1*-6His; this plasmid fully complemented a *far1* deletion strain) was grown in SD-URA to mid–logarithmic phase before expression of Far1, and Ste4 was induced by adding galactose (2%). Exposure of these cells to galactose induces the mating pathway because of overexpression of Ste4 (*28*) and allows production of Far1 in its fully phosphorylated and presumably active form (*6, 23*). As a control, we performed the same purification protocol with extracts prepared from cells that were not exposed to galactose and therefore did not produce Far1. After incubation for 5 hours with (or without) galactose, cells were centrifuged and extracts were prepared as described [M. Woonter, P. A. Wade, J. Bonner, J. A. Jaehning, *Mol. Cell. Biol.* **11**, 4555 (1991)]. The $(NH_4)_2SO_4$ pellet was resuspended in 20 mM Hepes-KOH (pH 7.5), 150 mM potassium glutamate, 10 mM $MgSO_4$, 10 mM EGTA, 20% glycerol, 5 mM PMSF, 2 mM benzamidine, 80 mM β-glycerophosphate, 10 mM NaF, and 5 mM dithiothreitol (DTT) and was dialyzed overnight against the same buffer at 4°C; portions were then frozen. The dialyzed extract was diluted in HNG binding buffer [20 mM Hepes (pH 7.8), 500 mM NaCl, 0.5% NP-40, 10% glycerol, 10 mM imidazole, 10 mM NaF, 60 mM β-glycerophosphate, 1% aprotinin, 0.1 mM PMSF, 2 mM benzamidine, 1 mM leupeptin] and applied to a column containing iminodiacetic acid immobilized on Sepharose-CLB (Sigma) coupled with Ni^{2+}. The column was washed with HNG buffer, and proteins were eluted with 10 ml of 0.5× HNG containing 200 mM imidazole. The eluate was diluted with 30 ml of HG buffer [20 mM Hepes-KOH (pH 7.5), 10 mM $MgSO_4$, 10 mM EGTA, 20% glycerol, 5 mM PMSF, 2 mM benzamidine, 80 mM β-glycerophosphate, 10 mM NaF, 5 mM DTT] and applied to a HiTrap Q column (Pharmacia). The column was washed extensively with HG buffer containing 100 mM NaCl, and proteins were eluted with HG buffer containing 500 mM NaCl. The eluate was diluted with HG buffer and applied to a HiTrap SP column (Pharmacia). The column was washed with HG buffer containing 100 mM NaCl, and bound proteins were eluted with HG buffer containing 500 mM NaCl. Fractions containing Far1-6His were then dialyzed against 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 2 mM PMSF, and 2 mM benzamidine, concentrated in a Centricon 30 microconcentrator (Amicon), and frozen in portions at −70°C.
22. Cdc28-Cln2 was immunoprecipitated from extracts prepared from YMP29 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL$^+$ psi$^+$ cln2::CLN2-HA-LEU2 far1::URA3*) as described (6). Immunoprecipitates were washed four times with buffer S (*20*) and twice with kinase buffer and divided into equal portions. One portion was tested for the presence of Cln2-HA by immunoblotting. Immunoprecipitates were incubated with purified Far1 for 30 min at 4°C in kinase buffer and then either washed gently with kinase buffer or used directly to assay H1 kinase activity as described (*20*). Phosphorylated histone H1 was excised from SDS-polyacrylamide gels and incorporated radioactivity was measured (Cerenkov counts). Full activity (100%) corresponds to counts per minute incorporated into histone H1 in reactions in which only buffer was added. Cdc28-Cln1, Cdc28-Clb2, and Cdc28-Clb5 complexes were immunoprecipitated with 12CA5 antibodies as described above from the following strains: YMT297 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL$^+$ psi$^+$ cln1::CLN1-HA*) (kindly provided by M. Tyers, University of Toronto; YMG5 (*MAT***a** *ade2-1 trp1-1::GAL-CLB2-HA-TRP1 can1-100 leu2-3,112 his3-11,15 ura3 GAL$^+$ psi$^+$*) (kindly provided by M. Glotzer, EMBL, Heidelberg); and K3819 (*MAT***a** *ade2-11 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3::ADH-HA-CLB5-URA3 GAL$^+$ psi$^+$*) (kindly provided by E. Schwob, IMP, Vienna).
23. The coding sequences corresponding to the NH_2-terminal 390 amino acids of the *FAR1* alleles 60F3 and 22 (starting from the corrected *Far1* NH_2-terminus [F. Cross, personal communication]) were cloned into the *E. coli* expression vector pGEX1 (Pharmacia). The GST fusion proteins were expressed in *E. coli* NB42 and purified by affinity chromatography on gluthathione–Sepharose 4B (Pharmacia) as described (*6*). Eluted fractions were dialyzed against 20 mM tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 2 mM PMSF, and 2 mM benzamidine, concentrated in a Centricon 30 microconcentrator, and frozen in portions. Concentrations of the purified GST-Far1 proteins were equalized (to approximately 5 μg/ml) after immunoblotting with antibodies to GST. Samples were used in H1 kinase assays as described (*22*).
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cDNA Cloning and Interferon γ Down-Regulation of Proteasomal Subunits X and Y

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Proteasomes are the proteolytic complex responsible for major histocompatibility complex (MHC) class I–restricted antigen presentation. Interferon γ treatment increases expression of MHC-encoded LMP2 and LMP7 subunits of the proteasome and decreases expression of two proteasome subunits, named X and Y, which alters the proteolytic specificity of proteasomes. Molecular cloning of complementary DNAs encoding X and Y showed that their proteins are proteasomal subunits with high amino acid similarity to LMP7 and LMP2, respectively. Thus, interferon γ may induce subunit replacements of X and Y by LMP7 and LMP2, respectively, producing proteasomes perhaps more appropriate for the immunological processing of endogenous antigens.

Cytosolic proteasomes, which function as an extralysosomal, adenosine triphosphate–dependent system for selectively degrading ubiquitinated proteins, are proposed to be involved in the processing of intracellular antigens into short peptides, which are then transported into the endoplasmic reticulum through the TAP1/2 heterodimeric peptide transporter (*1*). Two proteasomal polymorphic genes, LMP2 and LMP7, are located in the MHC class II region, closely linked to the TAP1 and TAP2 genes, and are up-regulated by interferon γ (IFN-γ), a major immunomodulatory cytokine (*2*). Treatment with IFN-γ induces a change in specificity of proteasomes for peptide degradation that is possibly responsible for effective generation of antigenic peptides associated with cell surface class I molecules (*3, 4*), but it is unknown how the specificity of proteasomes is changed. Not only is the synthesis of LMP2 and LMP7 induced, but the synthesis of two proteasome subunits, tentatively named X and Y, is reduced (*4*). To clarify the role of IFN-γ, we isolated and characterized cDNAs encoding X and Y.

For identification of proteasome subunits X and Y, the exact subunit composition of the human 20S proteasome was examined by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The enzyme from human kidney was separated into multiple components of different sizes and charges (Fig. 1). Protein spots corresponding to LMP2 and LMP7, which were identified as newly synthesized subunits that were induced by IFN-γ treatment, were detected by immunoblot analysis with anti-peptide antibodies to LMP2 and LMP7 (*4*).

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In contrast, two spots designated as X and Y, detected in a fluorogram of cell extracts metabolically labeled with ^{35}S-methionine that were then immunoprecipitated with an antibody to proteasomes, were found to be those of the subunits down-regulated in response to IFN-γ (*4*).

The X subunit generated four proteolytic fragments, after purification by high-performance liquid chromatography (HPLC) (*5*), and their amino acid sequences were determined. Full-length complementary DNA (cDNA) encoding X was then isolated by screening of a human HepG2 cell cDNA library by hybridization with a cDNA fragment synthesized by the polymerase chain reaction (PCR), on the basis of the amino acid sequences of these fragments. The amino acid sequence was predicted from the nucleotide sequence and was confirmed to be that of the X subunit of proteasomes by the fact that the sequences of the four fragments determined chemically (residues underlined in Fig. 2A) completely matched. Subunit X consists of 208 amino acid residues with a calculated molecular weight of 22,897, and its isoelectric point (pI) was calculated to be 8.67 by the method of Skoog and Wichman (*6*). These results are roughly consistent with the position of migration of X on 2D-PAGE (Fig. 1).

A proteolytic fragment of the Y subunit has the sequence FAVATLPPA (*5*), which matches the COOH-terminal sequence of the proteasomal δ that was partially sequenced (*7*). A monoclonal antibody to δ reacted specifically with spot Y on immunoblotting analysis after separation by 2D-PAGE (*5*). Thus, we concluded that subunit Y is identical to δ. We isolated the full-length cDNA encoding subunit Y (that is, δ) by screening the HepG2 cDNA library with a cDNA fragment synthesized by PCR, on the basis of the reported nucleotide sequence of δ. The polypeptide deduced from the open reading frame of the cDNA for Y consists of 239 amino acid residues with a calculated molecular weight of 25,315 and pI of 4.65 (Fig. 2B), which are consistent with its mobility on 2D-PAGE (Fig. 1).

Computer-assisted homology analysis showed that the primary structures of X and Y had significant similarities with those of other proteasome subunits, which themselves have high intersubunit similarities, but can be classified into two subfamilies with high similarities to the α and β subunit, respectively, of the archaebacterial proteasome (*8*). However, no significant similarities of these two proteins were found with those of other known proteins listed in the databases LASL-GDB (GenBank) and NBRF-PDB. Figure 3 shows results of statistical analyses of the similarities of human proteasomal subunits, including X and Y. So far, seven distinct cDNAs for α-type subunits, which show high similarity, have been isolated. Another nine subunits with less similarity to α-type subunits have been classified as β-type subunits. During evolution, the α-subunit family has been well conserved, whereas the β-subunit family may have diverged at an earlier stage. This divergence of β-type subunits may have been associated with the acquisition of specific functions for proteasomes. However, X and Y, both β-type subunits, were similar to the MHC-encoded proteasome subunits LMP2 (Y was 57% identical) and LMP7 (X was 68% identical, excluding the NH_2-terminal region) (Figs. 2 and 3). Thus, our data, together with observations that the expression of X and Y and of LMP7 and LMP2 changes reciprocally in response to IFN-γ (*4*), suggest that X and Y can be replaced by LMP7 and LMP2 in the proteasomal complex. This idea is consistent with recent reports that the subunit δ (that is, Y) may be replaced by LMP2 in response to IFN-γ (*9*, *10*). Interestingly, despite the high similarity in their primary structures, LMP7 has a calculated pI of 7.18, whereas X is a basic protein. This difference may affect the structural organization and hence functions of the proteasome. In contrast, Y and LMP2 are both acidic proteins with similar pIs of about 5.0.

Molecular genetic studies on yeast have shown that most proteasome genes are essential for cell proliferation (*11*). Interestingly, chromosomal deletions of the PRE2 and PRE3 genes, which may be homologs of

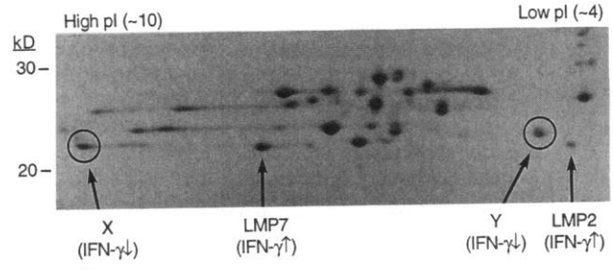

Fig. 1. Subunit pattern of human proteasomes. Proteasomes purified from human kidney were separated by 2D-PAGE and stained with Coomassie brilliant blue. The MHC-encoded proteasome subunits LMP2 and LMP7 that were up-regulated by IFN-γ were identified by immunoblot analysis (*4*). The two spots down-regulated by IFN-γ were designated as X and Y (*4*).

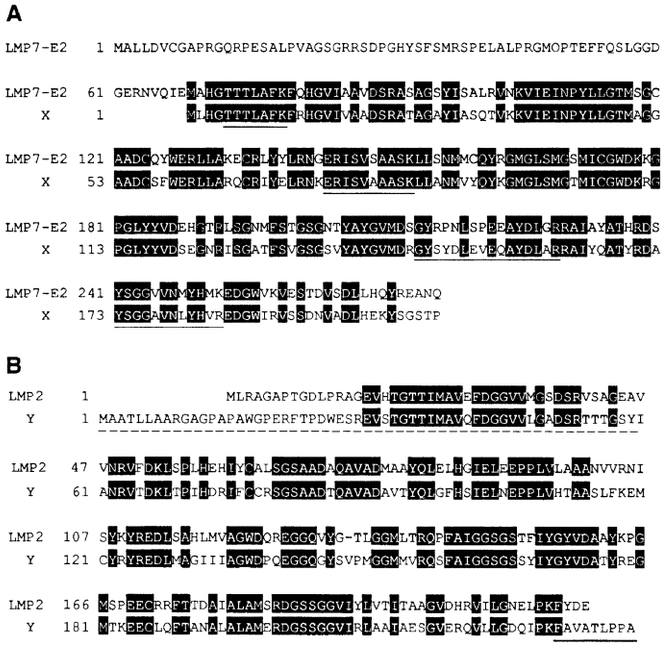

Fig. 2. Comparison of the amino acid sequences of X (**A**) and Y (**B**) with those of LMP7 and LMP2, respectively, from human proteasomes. The cDNA clones encoding subunits X and Y were isolated by screening of a human cDNA library (*16*). The predicted amino acid sequences were deduced from the nucleotide sequences. Continuous underlines in (A) show the amino acid sequences corresponding to those of four fragments of X determined by Edman degradation (*5*). The dotted line in (B) shows the amino acid sequence deduced from the isolated cDNA clone. Other regions were the same as in the amino acid sequence for δ reported by DeMartino *et al.* (*7*). The amino acid sequence of the COOH-terminal fragment of Y is underlined with a continuous thick line (*5*). Identical amino acids are boxed in black. Reported sequences of human LMP7-E2 (*2*, *17*) and LMP2 (*2*) are shown. Single letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequence data reported in this paper will appear in the Genome Sequence DataBase, DNA Data Bank of Japan, European Molecular Biology Laboratory, and National Center for Biotechnology Information Nucleotide Sequence Databases with the following accession numbers: D29011 for proteasome subunit X and D29012 for proteasome subunit Y.

	HC2	HC3	HC8	HC9	XAPC7	PROS-27	Zeta	HC5	HC7-I	HC10-II	HN3	MECL1	X	LMP7-E2	Y	LMP2
α-Type subunits																
HC2	100.0	30.7	22.6	28.0	32.8	23.1	28.9	13.1	15.7	16.6	16.1	18.8	17.5	15.8	15.3	14.3
HC3	29.4	100.0	28.7	38.1	37.2	34.8	32.0	16.6	17.9	15.2	15.0	18.8	20.3	19.2	15.9	14.3
HC8	23.2	30.0	100.0	25.0	29.3	28.8	31.9	12.5	12.6	11.1	15.6	15.2	13.5	11.2	12.4	15.2
HC9	27.2	35.8	26.2	100.0	34.2	26.6	33.0	22.0	17.6	16.2	17.0	24.6	21.1	18.3	16.7	16.8
XAPC7	32.2	36.2	29.7	35.0	100.0	31.9	35.0	15.7	16.2	16.3	15.0	24.0	17.6	17.6	20.6	20.7
PROS-27	22.7	33.6	29.3	31.0	31.9	100.0	23.1	19.2	18.3	15.1	14.4	19.2	16.9	15.7	16.3	19.2
Zeta	29.7	30.9	31.9	34.8	37.5	25.1	100.0	20.6	16.1	16.4	16.8	24.9	19.7	20.2	22.6	21.6
β-Type subunits																
HC5	10.9	14.5	11.2	20.0	14.0	15.8	17.5	100.0	21.0	24.9	22.5	21.4	21.8	21.3	20.8	20.4
HC7-I	15.8	16.2	13.1	16.0	15.2	16.6	15.7	19.9	100.0	16.7	22.1	21.5	20.2	22.0	16.8	22.1
HC10-II	16.2	13.6	10.9	14.4	15.4	12.9	15.2	25.6	16.6	100.0	18.0	17.2	21.2	20.0	21.2	21.3
HN3	13.4	13.8	12.5	14.2	12.4	11.6	13.7	18.1	20.3	18.3	100.0	19.9	24.9	28.8	19.3	19.4
MECL1	15.8	16.8	12.2	20.2	19.8	17.0	22.3	19.1	19.3	16.1	18.7	100.0	27.9	29.0	30.2	30.3
X	17.2	21.0	13.7	19.8	16.8	14.7	18.9	20.0	20.4	19.9	22.9	26.6	100.0	72.2	32.4	30.3
LMP7-E2	14.6	18.8	11.0	15.7	15.8	14.0	16.8	18.4	21.2	19.0	24.0	23.1	68.3	100.0	32.4	33.7
Y	13.7	15.8	12.3	14.0	17.5	14.9	19.1	18.3	17.2	19.5	18.3	25.0	30.4	26.6	100.0	63.1
LMP2	12.6	13.6	13.4	15.1	17.4	17.1	19.1	19.3	21.3	19.3	19.5	28.7	27.7	29.4	57.1	100.0

Fig. 3. Similarities of primary structures of various human proteasome subunits. The sources of sequence data are as follows: HC2, HC3, HC5, HC8, and HC9 (*18*); XAPC7 (*19*); PROS-27 (*20*); Zeta (*7*); MECL1 (*21*); HC7-I, HC10-II, and HN3 (*12*); LMP7-E2 (*17*); and LMP2 (*2*). Percentages of overall amino acid sequence identity between proteasomal subunits were obtained by computer analysis (boxed in black). The right side (open box) shows the percentage identity confined to highly conserved amino acid regions, which are HC2 (32–215), HC3 (32–213), HC8 (34–217), HC9 (31–216), XAPC7 (29–211), PROS-27 (36–220), Zeta (34–220), HC5 (37–218), HC7-I (6–181), HC10-II (8–192), HN3 (53–237), MECL1 (39–217), X (4–183), LMP7-E2 (72–251), Y (34–213), and LMP2 (20–198), in which numbers show amino acid residues (start-end) in the calculation. The gaps inserted to achieve maximal sequence homology of amino acids were deleted in statistical analyses.

LMP7 and LMP2, respectively, in budding yeast, are lethal (*12*), whereas human cells lacking the LMP7 and LMP2 genes grow normally, indicating that these genes are dispensable (*2*). This finding initially suggested that these subunits have different roles in different organisms. However, we found that the similarities of X and Y with PRE2 and PRE3 are approximately 68% and 54%, respectively, which are higher than the 54% similarity of LMP7 with PRE2 and the 44% similarity of LMP2 and PRE3. Thus, the actual homologs of PRE2 and PRE3 may be X and Y, not LMP7 and LMP2. It is unknown whether X and Y are essential genes.

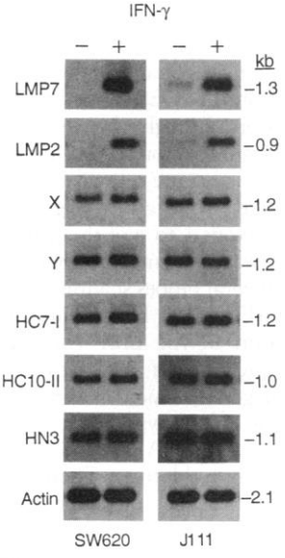

Fig. 4. Effect of IFN-γ on expression of the β-type proteasome subunit family in SW620 and J111 cells. Cells were treated with IFN-γ (500 U/ml) for 3 days (*4*), and then amounts of mRNAs encoding proteasomal proteins were analyzed by RNA blot hybridization (*22*). Molecular sizes are indicated on the right (in kilobases).

Interferon-γ treatment increases the mRNAs encoding LMP2 and LMP7, and the amounts of these two mRNAs roughly correlate with protein amounts detected by immunoblot analysis with their antibodies in various human cells (*4*). This finding suggests that proteasomes induced by IFN-γ are formed by de novo synthesis, possibly as a result of a change in transcription. However, the mRNA amounts of subunits X and Y were not affected by treatment with IFN-γ (Fig. 4), suggesting that the complete loss, or marked decrease in the expressions of subunit X and Y, is due to posttranscriptional regulation.

Interferon γ affects the proteolytic functions of proteasomes, enhancing their activities for endoproteolytic cleavage of peptide bonds on the carboxyl side of basic and neutral amino acid residues of proteins, but decreasing their activities for peptides containing acidic amino acid residues (*3*, *4*). In contrast, Boes *et al.* (*10*) reported reduction of the chymotrypsin-like activity by IFN-γ for some unknown reason. These changes of peptidase activities apparently correspond to the properties of antigenic peptides associated with cell surface class I molecules, the carboxyl sides of which consist mainly of hydrophobic and basic amino acid residues, not acidic amino acid residues (*13*). We have cloned cDNAs encoding the human proteasomal subunits HC7-I, HC10-II, and HN3, which are responsible for tryptic-, chymotryptic-, and V8 protease–like actions, respectively (*14*). Molecular genetic and biochemical studies (*15*) have suggested that these subunits or their yeast counterparts are involved in the respective proteolytic activities. Here we examined whether IFN-γ affects the expression of these genes (Fig. 4). Treatment with IFN-γ did not change the amount of mRNA for these peptide hydrolyses. This is consistent with our recent finding that the patterns of most newly synthesized proteasomal subunits, other than LMP2 and LMP7 and X and Y, are not altered by IFN-γ treatment (*4*). Thus, the acquisition of functional diversity of proteasomes induced by IFN-γ is due to changes of subunit assembly of proteasomes accompanying substitutions of LMP7 and LMP2 for X and Y, not to alterations in expressions of other genes, such as HC7-I, HC10-II, and HN3.

On the basis of the present findings, we propose that the basal processing of intracellular antigens is catalyzed by X-Y–type proteasomes. However, when large amounts of nonself antigens invade the cells, as during viral infection, these cytosolic antigens are efficiently processed by IFN-γ–induced proteasomes, which contain LMP7 and LMP2 instead of X and Y, and the peptides generated are transported into the endoplasmic reticulum through the TAP transporter system to be assembled with MHC class I molecules, which are also induced by IFN-γ. As reported (*4*), IFN-γ had no effect on the cellular content of proteasomes. Therefore, IFN-γ may modify the subunit organization and functions of proteasomes, which could result in processing of endogenous antigens. This is another mechanism for the immunomodulatory action of IFN-γ, differing from that of increase in the cellular concentrations of TAP transporter and MHC molecules responsible for antigen presentation by cells.

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16. A cDNA library of human hepatoblastoma HepG2 cells was constructed in a λZAPII phage expression vector (Stratagene). For isolation of cDNA for X, about 5×10^5 plaques were screened by hybridization with a cDNA fragment that had been synthesized by PCR and labeled with [α-^{32}P]deoxycytidine triphosphate. After plaque hybridization, the pBluescript plasmid was excised and directly sequenced by a double-strand strategy in an automatic DNA sequencer (Pharmacia LKB Biotechnology Inc.). For use as PCR primers, we selected parts of the sequences of two of the proteolytic fragments, YDLEVEQAYD and GGAVNLYHV, respectively, of subunit X (*5*). The following oligonucleotides corresponding to the protein sequences were synthesized: Forward primer, 5′-TATGATTTTGAGGTTGAGCAGGCTTATGA-3′; reverse primer, 5′-ACGTGGTATAGGTTTACTGCTCCTCC-3′. With these primers, a cDNA fragment of approximately 100 base pairs (bp) was synthesized by PCR with first strand cDNA complementary to mRNA from human hepatoblastoma HepG2 cells as a template. For isolation of cDNA for Y, a cDNA fragment of the δ subunit was synthesized by PCR on the basis of the reported sequence (*7*) and used as a probe, because we recently found that Y is identical to the δ subunit (*5*).
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22. Samples of 5 to 10 μg of total RNA were separated by electrophoresis in agarose gel containing formaldehyde, transferred to a Hybond-N$^+$ nylon membrane (Amersham), and hybridized with ^{32}P-labeled probes as described previously (*4*). For Northern (RNA) blot analysis of proteasomes, cDNAs for subunits HC7-I, HC10-II, and HN3 (*14*) of human proteasomes and the cDNA of β-actin (Oncor Inc.) were used. Approximately 300-bp cDNAs of LMP2 and LMP7 of human proteasomes were synthesized by PCR on the basis of reported sequences (*4*). These probes were labeled with a commercial kit for multiprime DNA labeling (Takara Shuzo, Kyoto). The membranes were washed and autoradiographed with Kodak XAR-5 film at −70°C with the use of an intensifying screen.
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MHC Class I Expression in Mice Lacking the Proteasome Subunit LMP-7

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Proteasomes degrade endogenous proteins. Two subunits, LMP-2 and LMP-7, are encoded in a region of the major histocompatibility complex (MHC) that is critical for class I–restricted antigen presentation. Mice with a targeted deletion of the gene encoding LMP-7 have reduced levels of MHC class I cell-surface expression and present the endogenous antigen HY inefficiently; addition of peptides to splenocytes deficient in LMP-7 restores wild-type class I expression levels. This demonstrates the involvement of LMP-7 in the MHC class I presentation pathway and suggests that LMP-7 functions as an integral part of the peptide supply machinery.

MHC class I molecules present peptides derived from endogenously synthesized proteins to CD8$^+$ T cells. These peptides are thought to be generated in the cytosol and transported into the endoplasmic reticulum (ER), where they associate with newly assembling class I molecules (*1*). None of the proteases involved in this process have been identified so far, but one candidate is the proteasome, a 700-kD complex that is abundantly present in the cytosol and nucleus of all cell types investigated. This large complex is composed of at least 13 to 15 distinct subunits, has several discrete proteolytic activities, and appears to constitute the major factor in nonlysosomal protein degradation of normal cellular protein turnover (*2*).

It has recently become clear that at least two proteasome subunits, LMP-2 and LMP-7, are encoded in the MHC class II region. These proteasome genes are closely linked to the genes encoding the putative peptide transporters TAP-1 and TAP-2 (*3*). This finding, supported by several indirect observations, has fueled the hypothesis that the MHC-encoded proteasome subunits may be components of the proteolytic machinery that provides antigenic peptides for presentation on class I molecules. Although in vitro studies with purified proteasomes and synthetic model substrates have lent some support to this hypothesis (*4*), definitive experimental evidence substantiating a role for LMP-2 or LMP-7 in antigen presentation is still lacking.

In order to better understand the physiological role of the MHC-encoded proteasome subunits, we generated a strain of mice lacking LMP-7 by means of homologous recombination in embryonic stem (ES) cells. To ensure complete inactivation of the gene encoding LMP-7, we used a deletion-type targeting vector (Fig. 1A). Upon homologous recombination, this construct was expected to eliminate exons 1 to 5, encoding the first 247 of the 276 amino acids of the LMP-7 protein. Of 72 G418 and gancyclovir double-resistant ES clones analyzed by Southern (DNA) blotting, 19 had undergone homologous recombination. Two independent clones, representing two discrete targeting events, were used to generate chimeric males that were mated with C57BL/6 or 129/Ola females. Chimeric founders from both ES clones transmitted the mutation through the germline, and heterozygous mice from each line were intercrossed to generate animals homozygous for the targeted mutation (Fig. 1B). The absence of exons 1 to 5 of the gene encoding LMP-7 was confirmed by Southern blotting with several probes spanning the deleted region (Fig. 1B). To examine whether the mutation in the LMP-7 locus might impair expression of the closely linked transporter genes, which in turn could influence the phenotype, we quantitated RNA coding for TAP-1 and TAP-2 by Northern (RNA) blotting. There was absolutely no difference in either TAP-1 or TAP-2 expression levels between mutant and wild-type mice (Fig. 1C), which excludes such a possibility.

Mice homozygous for the LMP-7 mutation bred well and appeared healthy in a conventional animal facility. They were indistinguishable from heterozygous or wild-type littermates on gross physical inspection

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