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The Interferon-γ-inducible 11 S Regulator (PA28) and the LMP2/LMP7 Subunits Govern the Peptide Production by the 20 S Proteasome *in Vitro**

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Marcus Groettrup‡, Thomas Ruppert§, Lothar Kuehn¶, Michael Seeger‡, Sybille Standera‡, Ullrich Koszinowski§, and Peter M. Kloetzel‡‖

From the ‡Institute for Biochemistry, Medical Faculty (Charité), Humboldt University Berlin, Hessische Strasse 3–4, D-10115 Berlin, the §Department of Virology, University of Heidelberg, Im Neuenheimer Feld 324, D-69120 Heidelberg, and the ¶Diabetes Research Institute, Auf'm Hennekamp 65, D-40225 Düsseldorf 1, Federal Republic of Germany

Antigenic peptides presented on major histocompatibility complex (MHC) class I molecules to cytotoxic T cells are generated in the cytosol by the 20 S proteasome. Upon stimulation of antigen presenting cells with interferon-γ, two constitutive subunits of the 20 S proteasome are replaced by the MHC-encoded subunits low molecular mass polypeptide (LMP) 2 and LMP 7. In addition the expression of the two subunits of the 11 S regulator of the 20 S proteasome (PA28) are increased. As the function of LMP2 and LMP7 in antigen presentation is still controversial, we tested whether these subunits might operate by modifying proteasome activation through the 11 S regulator. We strongly overexpressed the two LMP subunits separately or together by transfection in murine fibroblasts. Isolated 20 S proteasomes from LMP transfectants were applied in digests of a 25-mer peptide in the presence or absence of a purified preparation of 11 S regulator from rabbit erythrocytes. Analysis of the cleavage products by high performance liquid chromatography and electrospray mass spectroscopy revealed marked differences in the peptide product profile in dependence on the LMP2 and LMP7 content. While the 11 S regulator did not preferentially activate LMP2 or 7 containing proteasomes, the binding of the 11 S regulator to any of the proteasome preparations markedly changed both the quality and quantity of peptides produced. These results suggest that the 11 S regulator increases the spectrum of peptides which can be generated in antigen presenting cells.

In the course of a viral infection, the production of antigenic peptides from intracellular viral proteins has to meet high demands: in order to fit into the groove of major histocompatibility complex (MHC)[1] class I molecules these peptides need to have a defined length of 8 or 9 residues including fixed amino acids as anchor residues. For the analyzed murine and human MHC haplotypes the C terminus is either an aliphatic (Leu, Val, Ile), aromatic (Tyr), or basic (Arg, Lys) residue (1, 2). A further fixed consensus amino acid lies in position 2 or 5, and even residues at nonanchor sites are not arbitrary (3). The viral peptide has to meet the less stringent selectivity of TAP transporters (4), and it cannot cross-react with a self-peptide for T cell receptor binding as the T cells of that specificity are eliminated during negative selection in the thymus (5).

There is increasing evidence that the 20 S proteasome, also called multicatalytic proteinase, is responsible for generating antigenic peptides. The 20 S proteasome is the major cytosolic endoprotease in eukaryotes (6–8). These 700-kDa protease complexes, which constitute 0.5–1% of total cell protein, consist of 14 different subunits ranging in molecular mass from 21 to 32 kDa and with isoelectric points from 3 to 10, as evidenced by two-dimensional analysis on NEPHGE-PAGE gels (9). The subunits can be classified as α and β type based on their homology to the two different subunits, α and β, of an ancestral proteasome found in the archaebacterium *Thermoplasma acidophilum* (10). Seven α and seven β subunits each form two rings stacked in the order α-β-β-α to build the cylinder-shaped complex. Among the β type subunits, LMP2 and LMP7, which are encoded in the vicinity of the peptide transporter genes in the MHC II complex (11–14), are induced by the stimulation of cells with interferon-γ, and they replace their constitutive counterparts, designated delta and MB-1, in the complex (15–18).

Two further genes which are up-regulated by IFN-γ are not part of the 20 S proteasome itself but encode the two subunits constituting the "11 S regulator" (REG) or "PA 28" which is a potent activator of the 20 S proteasome (19–24). Freshly isolated 20 S proteasomes are "latent" when assayed with tri- or tetrameric standard fluorogenic peptide substrates. Depending on the N-terminal amino acid from which a fluorescent leaving group like MCA is cleaved by the proteasome, the REG activates the proteasome 20–50-fold (Suc-LLVY-MCA, (*Z*)-LLE-βNA), 10-fold (PFR-MCA), or 3-fold (GGF-MCA). The REG has been isolated from human blood as a hexa- or heptameric 180-kDa particle consisting of two subunits with apparent molecular weights of 29 and 31 in SDS-PAGE. In evolution both subunits are higly conserved with about 90% amino acid sequence identity between human and rat. The 29- and 31-kDa subunits may be functionally different as they display only about 50% identity between each other (24, 25). Electron microscopy has shown that the ringshaped REG binds to the α-end plates of the proteasome (26). It thus competes with another complex activator, called the 19 S regulator, for binding to the 20 S proteasomes (27). However, in contrast to the reversible association between REG and 20 S proteasome

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‖ To whom correspondence should be addressed. Tel.: 030-284-68-539; Fax: 030-284-68-217.

[1] The abbreviations used are: MHC, major histocompatibility complex; DTE, dithioerythritol; HPLC, high performance liquid chromatography; LMP, low molecular mass polypeptide; MCA, 7-amido-4-methylcoumarin; NEPHGE, nonequilibrium pH gradient electrophoresis, PAGE, polyacrylamide gel electrophoresis; pNA, *p*-nitroanilide; REG, 11 S regulator; Suc, succinyl; TAP, transporter associated with antigen presentation; (*Z*), benzyloxycarbonyl; IFN, interferon; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; PCR, polymerase chain reaction; FPLC, fast protein liquid chromatography.

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which is energy independent, formation of the 26 S proteasome out of the 20 S proteasome and the 19 S regulator is ATP-dependent (28). At least four of the 13–15 subunits of the 19 S regulator belong to a novel family of ATPases, and one subunit has been shown to be the receptor of ubiquitin, crucial for the function of the 26 S protease in degrading ubiquitinated proteins (29).

Interferon-γ potentiates antigen presentation on MHC class I molecules (30) by increased transcription of MHC class I and TAP genes. The finding that two subunits of the proteasome and of the REG are IFN-γ inducible is a strong indication that the proteasome is involved in the production of antigenic peptides. In fact, proteasome inhibitors prevent an *in vivo* production of peptide ligands for MHC class I molecules from ovalbumin (31). Proteins or peptides cleaved *in vitro* by the 20 S proteasome have been found to yield antigenic peptides (32, 33). Mice deficient for the LMP7 gene show a decrease in MHC class I surface expression on lymphocytes and the *in vitro* stimulation of HY-antigen-specific T cells was reduced (34). LMP2-deficient mice, in contrast, are not reduced in MHC class I expression but show a diminution of $CD4^-8^+$ T cells. Upon infection with influenza A virus, these mice show a reduction in the frequency of precursors of antigen-specific cytotoxic T cells while no change in cell number was noted in Sendai virus infection (35). It appears that the presence of LMP2 and LMP7 subunits in the 20 S proteasome is not required for MHC class I expression and antigen presentation (36, 37) but some viral antigens are presented more efficiently.

How LMP2 and LMP7 mediate these effects on antigen presentation remained a controversial issue. In some laboratories *in vitro* experiments with fluorogenic peptides and proteasomes from an LMP2/LMP7 doubly deficient lymphoblastoid cell line yielded a reduction in cleavage at the C terminus of tyrosine and arginine residues as compared to wild type (38, 39), whereas this was not found by other investigators (32, 40). We have therefore readdressed this issue by strongly overexpressing LMP2 and LMP7 alone or together in transfected murine fibroblast cells. We further tested how the REG would influence these proteasome populations in *in vitro* digests of a 25-mer peptide. While LMP2 and LMP7 caused substantial variation in the quantity of different peptides produced, binding of the REG to any of these 20 S proteasome preparations led to a characteristic qualitative and quantitative shift in the cleavage products generated.

MATERIALS AND METHODS

Cell Culture and Transfections—C4 is a murine fibroblast line derived from embryonic BALB/c mice by SV40 infection *in vitro*.[2] The B8 clone had been derived by cotransfection of the C4 line with plasmid pIE100, an expression construct encoding the pp89 protein of the murine cytomegalovirus (41), and pAG60 encoding the neomycin resistance gene. B8 cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin/streptomycin, 250 μg/ml G418. B8 cells were transfected with plasmids encoding BALB/c-derived LMP2 or LMP7 full-length cDNAs cloned into pSG5 (Stratagene, Heidelberg, Germany) by direct cloning using *Eco*RI sites for LMP7 (42) and PCR cloning using *Bam*HI/*Bgl*II sites and primers as described for LMP2 (15). Cells (5×10^5) plated to 60% confluence were transfected by standard calcium phosphate precipitation methods with 8 μg of pLMP2 or 8 μg of pLMP7 or 4 μg of each in cotransfection with 2 μg of either puromycin (pLXSP) or hygromycin (pLXSH) resistance expression constructs (generous gifts of Dr. Ed Palmer, Basel, Switzerland). One day after transfection cells were replated in 96-well plates under cloning conditions and selected after 2 days in 2.5 μg/ml puromycin or 400 μg/ml hygromycin.

Purification of the 11 S Regulator—The detailed purification procedure will be documented in a separate publication.[3] In brief, rabbits (source: SAVO-Ivanovas GmbH, Kisslegg, Germany) were bled, and blood was collected in anti-coagulant solution. All subsequent steps were performed at 6 °C. Following four washes with 0.9% NaCl and centrifugation, packed cells were lysed with 3 volumes of TEAD buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, 1 mM DTE, pH 7.5) and cell debris was spun down ($80,000 \times g$, 60 min). The resulting supernatant was batch-adsorbed to DEAE-cellulose (DEAE-Servacel, Serva, Heidelberg), and the gel was washed with TEAD buffer. Elution of proteins containing REG was done with TEAD buffer containing 0.5 M NaCl. This step was followed by fractionation with ammonium sulfate (0–40 and 40–70% saturation with respect to the salt. The precipitate resulting from 40 to 70% salt cut was dissolved in TEAD buffer and dialyzed against the same buffer. Further enrichment of REG was achieved by consecutive anion-exchange chromatography on a DEAE-Sephacel and FPLC®-MonoQ column (both from Pharmacia, Freiburg). Purification to apparent homogeneity was performed by hydrophobic interaction chromatography on an FPLC®-phenyl-Superose column, yielding a preparation which showed a single band of about 200 kDa in native PAGE, and which is resolved into the two constituent subunits of 29 and 31 kDa upon SDS-PAGE (see Fig. 3). When stored at 6 °C in TEAD buffer, supplemented with 1 volume of glycerol, REG protein purified according to this protocol was found to retain full activity over a period of at least 8 weeks.

Purification of 20 S Proteasomes—Frozen pellets of 4×10^8 B8 cells were lysed in 10 ml of lysis buffer (80 mM KAc, 5 mM $MgAc_2$, 10 mM HEPES, pH 7.2, 0.1% Triton X-100) on ice and homogenized in a Dounce homogenizer. The $40,000 \times g$ supernatant of the lysate was bound for 45 min to DEAE-Sephacel (Pharmacia), unbound protein was removed by washing with buffer A (80 mM KAc, 5 mM $MgAc_2$, 10 mM HEPES, pH 7.2), and protein was eluted with buffer B (500 mM KAc, 5 mM $MgAc_2$, 10 mM HEPES, pH 7.2). Protein containing fractions were concentrated on a concentrator (Amicon Corp.) and loaded on a 10–40% sucrose gradient in buffer A. After centrifugation at 40,000 revolutions/min in a Beckman SW40 Ti rotor for 15.49 h, gradient fractions were tested for protease activity and active fractions pooled and concentrated for FPLC® chromatography on a MonoQ HR5/5 column (Pharmacia). A linear gradient was developed with buffers C (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2) and D (1 M KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2) and a flow rate of 1 ml/min. The 20 S proteasome eluted at 29% D as a single peak upon rechromatography and was >90% pure as judged by Coomassie-stained PAGE gels. Quantification of native proteasome protein was done by UV absorbtion at 280 nm using an extinction coefficient of 1.0 cm^2/mg. A typical yield was 200 μg of proteasome/4×10^8 cells.

Peptide Analysis by Mass Spectrometry—20 μl of proteasome digest were separated by reverse phase HPLC (SMART-system equipped with a μRPC C2/C18 SC 2.1/10 column (Pharmacia, Freiburg, Germany). Eluent A, 0.1% trifluoroacetic acid; eluent B, 70% acetonitrile containing 0.09% trifluoroacetic acid. Gradient 20–65% B in 32 min, flow rate 50 μL/min) and analyzed on-line by a tandem quadrupole mass spectrometer TSQ 7000 (FInningan MAT, Bremen, Germany) equipped with an electrospray ion source. Each scan was acquired over the range m/z 200-1700 in 3 s. The peptides were identified by their molecular mass calculated from the m/z peaks of the single or multiple charged ions. Additionally, the amino acid sequences of the major cleavage products were determined in MS/MS experiments from the fragmentation spectra after collision-induced dissociation with argon atoms.

Protease Assays and HPLC Separation—Fluorogenic substrate peptides Succ-LLVY-MCA, (*Z*)-GGL-MCA, VGR-MCA (Bachem, Heidelberg) were prepared from 10 mM stocks in Me_2SO and incubated at several final concentrations with 100 ng of purified proteasome in 100 μl of buffer E (50 mM Tris-HCl, pH, 7.5, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA) at 37 °C for 30 min, and the reaction was stopped by addition of 10 μl of buffer F (30 mM NaAc, pH 4.3, 100 mM $CH_2ClCOOH$, 70 mM acetic acid); fluorescence was determined with a RF-5001 PC spectrofluorometer (Shimadzu) at 380 nm excitation/440 nm emission. The (*Z*)-LLE-βNA substrate was dissolved freshly for each experiment, the reaction stopped by addition of 1 volume of ethanol, and the measurement was performed at 335 nm excitation/410 nm emission.

For digestion of a synthetic 25-mer peptide derived from the sequence of Murine Cytomegalovirus pp89 IE protein, 20 μg of peptide (kindly provided by Dr. P. Henklein, Berlin) were dissolved in 300 μl of buffer G (30 mM Tris-HCl, pH 7.5, 10 mM KCl, 0.5 mM DTE) and digested with 1 μg of purified proteasome for indicated times at 37 °C. REG was added at 8-fold molar excess in TEAD buffer + 1 volume of glycerol, and the negative controls were supplemented with TEAD/glycerol buffer only. Cleavage products were analyzed by reverse phase

[2] U. Koszinowski, unpublished results.
[3] L. Kuehn and B. Dahlmann, manuscript submitted for publication.

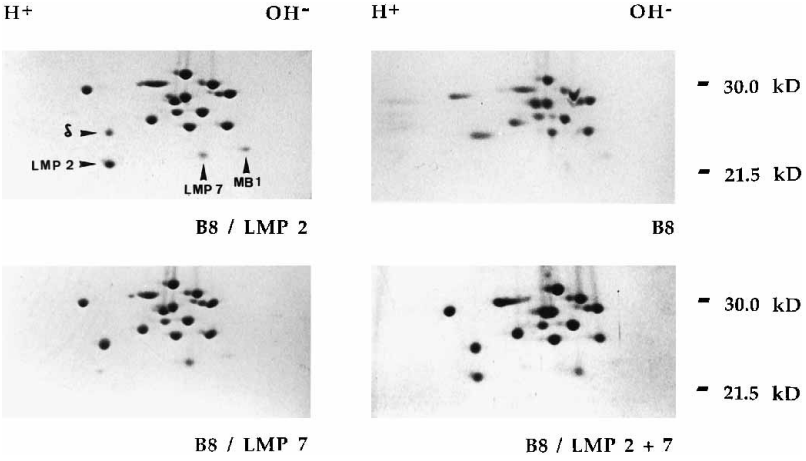


FIG. 1. **Two-dimensional NEPHGE/PAGE gels of 20 S proteasomes purified from LMP2- and LMP7-transfected B8 cells.** 20 S proteasomes were isolated from B8 wild-type cells and transfectant clones BC2P6 (*B8/LMP2*), B7H6 (*B8/LMP7*), BC27H7 (*B8/LMP2+7*). We applied 50 μg of protein to each gel as detailed under "Materials and Methods." Note the exchange of LMP2 for δ and LMP7 for MB1 (labeled with *arrows* in the *upper left panel*) in the respective transfectants. These preparations were used for all subsequent experiments.

HPLC: 50 μl of digest was applied to a 4.6 × 250 mm Ultrasphere RP18 column (Beckman) on a System Gold (Beckman) and eluted with a flow rate of 0.5 ml/min and a linear gradient of solution A (water, 0.1% trifluoroacetic acid) and solution B (acetonitrile, 0.1% trifluoroacetic acid): 0–5 min 0% B, 5–40 min linear increase to 60% B, peaks were detected at 220 nm.

NEPHGE-PAGE Two-dimensional Gels—Trichloroacetic acid precipitates of 50 μg of proteasome were agitated overnight in 60 μl of NEPHGE sample buffer (9.5 M urea, 2% Nonidet P-40, 5% ampholines, pH 3–10, (Servalyt, Serva), 0.3% SDS, 5% β-mercaptoethanol). Gel rods were poured as described (43) by addition of 27 μl of 10% ammoniumpersulfate and 19 μl of TEMED to a filtered and degassed solution of 5.5 g of urea in 1.32 ml of acrylamide stock (28.38% acrylamide, 1.62% bisacrylamide), 4.0 ml of 5% Nonidet P-40, 0.5 ml of Servalyte 3–10. The gel was topped up with overlay solution (8 M urea, 2, 5% Servalyt 3–10) and polymerized for 1 h. The sample was applied to the gel, topped with 20 μl of overlay solution, and run for 4 h at 400 V from acidic (0.01 M H_3PO_4, plus pole) to the basic side (0.02 M NaOH, minus pole). The rod was equilibrated for 45 min in 25 ml of equilibration buffer (10% glycerol, 10% β-mercaptoethanol, 2.3% SDS, 90 mM Tris-HCl, pH 6.8) and fixed to the top of a 15% SDS-PAGE with Laemmli SDS sample buffer + 1% agarose. The gels were run for 1050 V/h and stained with Coomassie stain.

RESULTS

Overexpression of LMP2 and LMP7 in Fibroblasts—B8, a fibroblast line derived from a BALB/c mouse, was shown before to express low endogenous levels of LMP2 and LMP7 proteins in isolated 20 S proteasomes in the absence of IFN-γ stimulation (32). We mimicked the IFN-γ-mediated induction of these two subunits by single or joint constitutive overexpression of LMP genes in B8 cells which permits the analysis of each subunit independent of other IFN-γ-mediated effects. Full-length cDNAs encoding LMP2 and LMP7 from a BALB/c derived library had been cloned by PCR into the pSG5 expression vector (15, 44), and the constructs were cotransfected with either hygromycin or puromycin resistance vectors under clonal conditions. Out of 20 drug resistant clones analyzed by genomic PCR, we have obtained 76% positive clones in LMP2 transfection, 85% positive clones in LMP7 transfection, and 57% LMP2/LMP7 double positive clones in the double transfection experiment, suggesting that LMP2 and LMP7 overexpression neither inhibits cellular growth nor survival. Virtually all PCR positive clones overexpressed the respective LMP proteins in Western blots as compared to untransfected B8 cells, albeit at different intensity. The LMP2 and LMP7 reactive antisera (15) detected proteins of 24 and 21 kDa for LMP2 and 30 and 23 kDa for LMP7 which are the molecular masses of the precursor and mature proteins, respectively. Thus, the expression and processing of overexpressed LMP subunits is normal.

A representative clone out of the LMP2 (BC2P6), the LMP7 (B7H6), and double transfectants (BC27P7) were raised in bulk culture, and 20 S proteasomes were purified. Analysis of the subunit pattern on two-dimensional NEPHGE-PAGE gels (Fig. 1) convincingly documents the overexpression of LMP2 and LMP7 as well as an extensive replacement of subunit δ by LMP2 and MB1 by LMP7, respectively. Except for this exchange no other consistent alterations in the two-dimensional pattern of the 20 S proteasome were noted. The significant acidic shift of the subunit C8 (30 kDa, basic of delta) in the double transfectant might be due to phosphorylation (45), but as it was not observed in a second preparation we did not further investigate this issue. The size and isoelectric point of the overexpressed LMP2 and LMP7 proteins in single and double transfectants are identical to those of the endogenously expressed proteins. Overexpression of one LMP subunit does not affect the endogenous expression of the other LMP subunit. Thus, in contrast to what has been suggested by other investigators (46), in our system LMP2 and LMP7 do not need each other nor any further IFN-γ-inducible factor for incorporation into the 20 S proteasome.

Proteasome Activation Through the 11 S Regulator Is Not Influenced by LMP Subunits—We used freshly prepared 20 S proteasome preparations from B8 transfectants to assess the impact of LMP subunits on the cleavage of fluorogenic peptides with F, R, L, Y, or E at the C-terminal P-1 position. The results, summarized in Table I, reveal two marked effects. First, proteasomes isolated from an LMP2/LMP7 double-transfectant cleaved the substrate Suc-LLVY-MCA at a significantly reduced rate when compared to those from wild-type B8 cells or single transfectants. Second, proteasomes purified from LMP2 or LMP2+7 transfectants cleaved the substrate (*Z*)-LLE-βNA much less efficiently than those derived from B8 cells or LMP7 transfectants. A kinetic analysis performed over a broad range of substrate concentrations has confirmed these findings (data not shown). The same changes in cleavage-site preference have been observed following similar transfection experiments in T2 lymphoblastoid cells (47) and after induction of RMA T cells with IFN-γ.

The evidence that LMP2 and LMP7 exert their effects by altering the cleavage pattern produced by the 20 S proteasome has largely been derived from experiments using short fluorogenic peptides. Using these substrates, the results reported by different investigators may differ dramatically from each other and even appear contradictory (32, 38–40, 46). Therefore, we set up experiments to test whether LMP2 and LMP7 possibly operate via proteasome activation by the 11 S regulator. As the material for regulator isolation is very limited in mice, we chose rabbit erythrocytes as a more abundant source. When analyzed by SDS-PAGE, regulator protein purified to apparent homogeneity (and used in all subsequent experiments) is resolved into two closely migrating bands of 29 and 31 kDa (Fig. 2) which

correspond to the two constituent subunits of the native regulator molecule.

In order to establish the amount of REG required for maximally stimulating 20 S proteasomes from wild-type cells and LMP transfectants, we have measured the cleavage activity toward Suc-LLVY-MCA as substrate. Maximum activation of proteasomes was achieved at an 8-fold molar excess of REG. Maximal activation by REG was about 15-fold in all proteasome preparations tested. Thus, at least in this system, there is no evidence that proteasomal LMP2/7 content affects activation factors or equilibrium constants of REG-proteasome binding. A kinetic analysis of Suc-LLVY-MCA hydrolyzing activity in the presence and absence of REG confirmed that the reduction of activity observed following overexpression of LMP2 and LMP7 (Table I) is not compensated by binding of the REG (data not shown).

The REG Changes the Priority of Cleavage Sites in the Course of a 25-mer Peptide Digest—The 20 S proteasome degrades larger peptides and a number of proteins to produce smaller peptides with a length of four to about 15 amino acids (32, 48). For that reason it is doubtful whether proteasomal cleavage characteristics can be derived from experiments examining the C-terminal cleavage of fluorogenic tri- and tetrapeptides. So far, the activation of 20 S proteasomes by the REG has been studied by using such fluorogenic peptides only. It therefore appeared of particular interest to investigate whether binding of the REG might cause changes in the spectrum of peptide products generated from a more physiological substrate. The substrate used, a synthetic 25-mer polypeptide, has a sequence corresponding to the amino acids 162–186 of the murine cytomegalovirus major immediate early protein pp89 (49) which contains a nonameric immunodominant T cell epitope (50).

The 25-mer peptide was incubated with 20 S proteasomes purified from B8 cells in the presence or absence of an 8-fold molar excess of REG from rabbit erythrocytes. Aliquots were withdrawn at indicated times and analyzed by reverse phase HPLC. The peptide region of the HPLC profiles generated with or without REG are shown in Fig. 3. In digests where 25-mer is exposed to B8 proteasomes in the absence of REG, a time-dependent change in the cleavage profile of the peptide is observed, and only at 48 h of incubation is this profile stable. In contrast, in the presence of REG, the same effect is observed at a much earlier time, that is 10 h after starting the incubation. The intact 25-mer is primarily cleaved to yield a dominant intermediate product after 5 h of digest while in the presence of REG many additional cleavage products of comparable peak magnitude are seen. To test whether kinetic differences might be due to inactivation of proteasome or REG, aliquots were removed after 24 and 36 h of incubation and activity toward Suc-LLVY-MCA was tested. The results (not shown) revealed no loss of activity in proteasome + REG incubations, while in experiments without REG, proteasome activity was even enhanced after 24 h. As an important control the 25-mer was incubated with our REG preparation alone since it cannot be completely ruled out that the REG might be proteolytically active itself. The 25-mer was not processed after 10 h of incubation at 37 °C, and only negligible degradation is seen after 24 h. Thus, we conclude that the REG is not an active protease by itself. Our peptide profiles illustrate that the REG accelerates the digest considerably, that it strongly influences the priority of initial cleavages, and that qualitatively different peptides are generated.

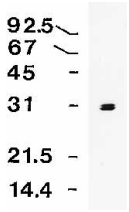


FIG. 2. **SDS-PAGE of REG isolated from rabbit erythrocytes.** Purified REG (2 μg) was subjected to SDS-PAGE on a 10–20% (w/v) acrylamide continuous gradient and electrophorized as detailed elsewhere (55). The larger and smaller REG subunit migrate to a position corresponding to a molecular mass of about 31 and 29 kDa, respectively. Standard proteins of known molecular mass are: phosphorylase *b* (92.5 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

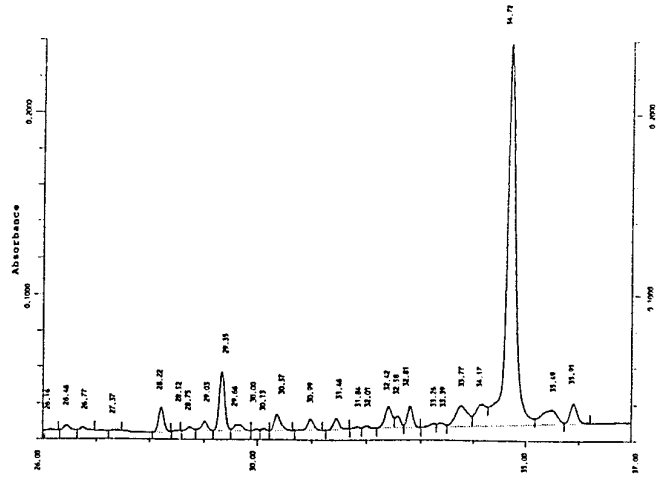
Combinations of LMP2, LMP7, and REG Contribute to Peptide Diversity—In order to compare the impact of REG binding and LMP incorporation on the generation of proteasomal cleavage products, we digested the pp89 25-mer model peptide with 20 S proteasomes isolated from B8 (wild-type), B7H6 (LMP7 transfectant), BC2P6 (LMP2 transfectant), and BC27H7 (LMP2+7 transfectant) in the absence or in the presence of an 8-fold excess of REG protein. Each of these digests was performed to completion that is for 48 h in the absence and for 24 h in the presence of REG and were found to yield highly reproducible results even with independent preparations of proteasomes. The peptide products were separated by HPLC, and fragments of the 25-mer polypeptide were identified via determination of their mass by electrospray mass spectrometry. Fig. 4 displays the amount of five selected peptide fragments produced in this experiment, and the ion currents of 11 peptides obtained by mass spectrometry, which are directly proportional to the quantity of peptide generated, are listed in Table II. No significant amount of 25-mer peptide was detected in any of the digests indicating a complete turnover of the

TABLE I

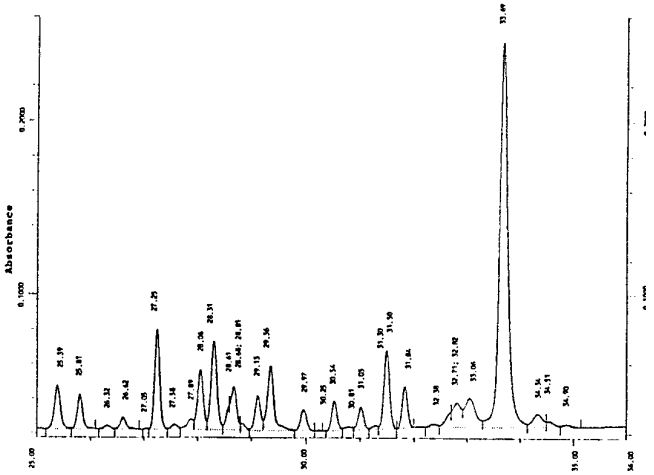
Hydrolysis of fluorogenic peptides by 20 S proteasomes from LMP transfectants

The hydrolytic activity of isolated proteasomes from indicated transfectants is measured by the production of free-fluorescent leaving groups (MCA or β-NA) and displayed in units of nmol substrate/h·10 μg proteasome. Results are the means ± S.E. for $n = 3$ assays. 100 ng of proteasome were applied in 30 min digests of indicated fluorogenic peptides at a concentration of 100 μM and an assay volume of 100 μl.

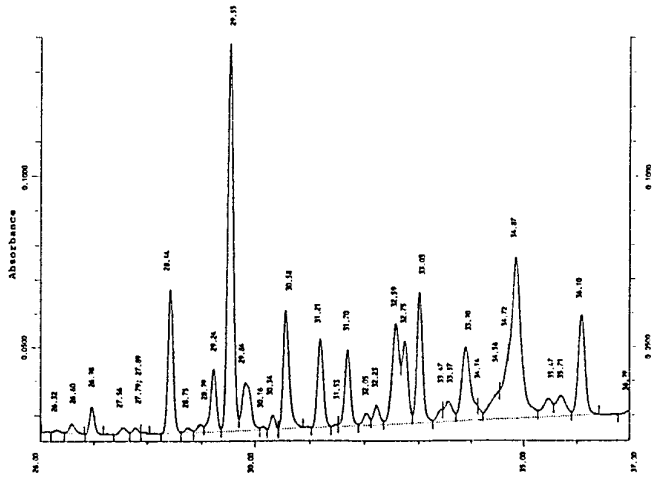
Name of cell line (genes transfected)	Fluorogenic substrate				
	Suc-AAF-MCA	Bz-VGR-MCA	(*Z*)-GGL-MCA	Suc-LLVY-MCA	(*Z*)-LLE-βNA
B8 (B8)	0.48 +/−0.03	0.89 +/−0.08	4.97 +/−0.30	8.35 +/−0.82	2.37 +/−0.17
BC2P6 (B8 + LMP2)	0.49 +/−0.04	0.97 +/−0.04	3.76 +/−0.08	10.13 +/−0.33	0.99 +/−0.05
B7H6 (B8 + LMP7)	0.47 +/−0.01	1.14 +/−0.17	4.41 +/−0.12	7.89 +/−0.27	3.08 +/−0.65
BC27H7 (B8 + LMP2+7)	0.50 +/−0.02	1.28 +/−0.13	5.96 +/−0.22	5.11 +/−0.44	1.35 +/−0.13



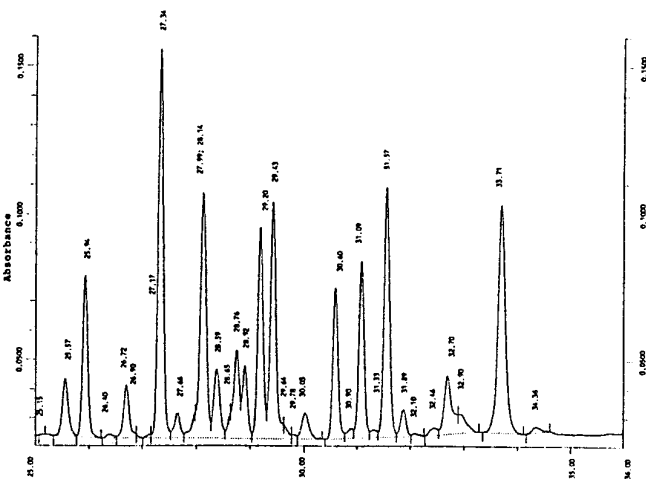
B8 1.5 h



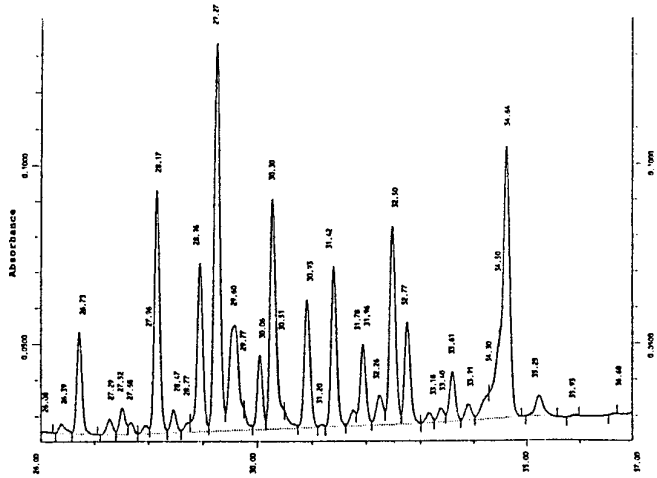
B8 + REG 1.5 h



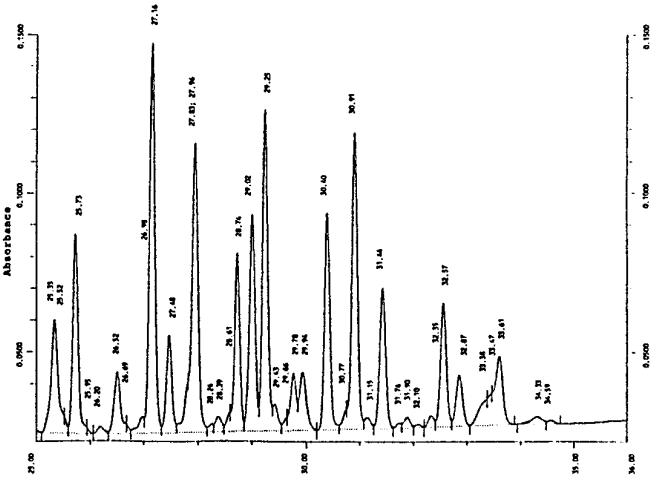
B8 5 h



B8 + REG 5 h



B8 24 h



B8 + REG 24 h

FIG. 3.

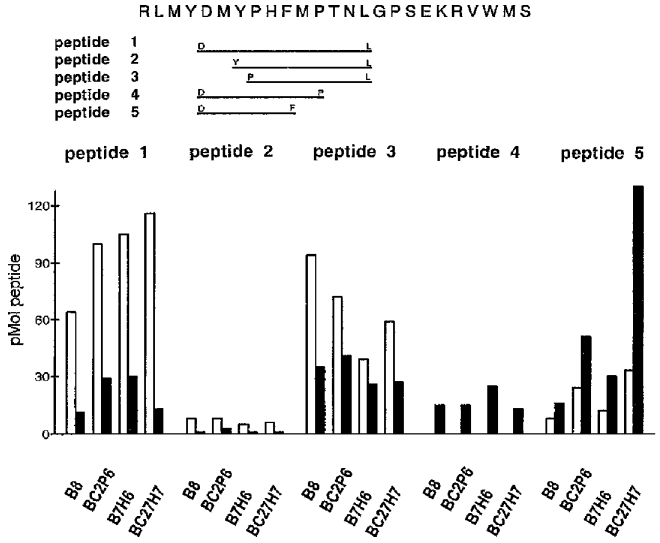


FIG. 4. **Amounts of five selected peptide fragments produced by *in vitro* digest from a 25-mer polypeptide.** 20 S proteasomes isolated from B8 wild-type cells and transfectants BC2P6 (B8/LMP2), B7H6 (B8/LMP7), BC27H7 (B8/LMP2+7) were used to digest a 25-mer peptide (sequence shown) in the presence (*closed columns*) and absence (*open columns*) of REG. The sequences of the five selected peptides is displayed above; note that peptide 2 is the immunodominant nonamer. Digests were performed to completion that is for 48 h in the absence and for 24 h in the presence of REG as described under "Materials and Methods." Peptide products were separated on HPLC and identified by their mass/charge values in mass spectrometry. The relative amounts were derived from the ion current shown in Table II. The absolute amount of peptides were calculated from the absorbance of UV light at 214 nm. For calibration the absorbance of three synthetic peptides (4, 9, and 11 amino acids long) were correlated with their number of peptide bonds. □, −REG; ■, +REG.

substrate. The variations in the amount of generated peptides between the LMP transfectants were in the order of 2–5-fold. Different amounts of peptides were obtained in dependence on incorporation of LMP2 alone, of LMP7 alone, or of the two subunits together, and the effects of LMP2 and LMP7 were neither additive nor following any obvious rule. The most marked difference was observed in the case of the single LMP2 transfectant where the RLMYD peptide was undetectable, and the amount of the RVWMS peptide was decreased 5-fold as compared to wild-type. We did not see a consistent reduction of the cleavages C-terminal of tyrosine following coexpression of LMP2 and LMP7 as would be predicted from our data obtained with fluorogenic peptides (Table I). Moreover, cleavage at the C termini of the two tyrosine residues contained in the 25-mer polypeptide was affected differently by LMP subunit expression as can be concluded from a comparison of the amounts of peptides 1 and 3 which have the same C terminus. We conclude that the hydrolysis of short fluorogenic peptides does not adequately describe the cleavage of peptide bound in larger peptides by the 20 S proteasome and that the decision on the cleavage site, which is strongly influenced by the incorporation of LMP subunits, is not simply dependent on the residue in the P1 position.

Strikingly, when the REG was included in the digests, the peptide profile generated by all proteasome preparations changed in a characteristic manner. This change was quantitative as well as qualitative since several peptides were newly generated (*e.g. peptide 4* in Fig. 4) while other peptides disappeared (*e.g.* the MYPHFMPTNL peptide in Table II). In the presence of REG, quantitative differences between the amount of peptides produced by proteasomes with different LMP content are still observed, indicating that REG binding does not compensate LMP-mediated cleavage preferences. Again our data fail to confirm a preferential activation of cleavage after tyrosine following the binding of the REG to the 20 S proteasome as has been deduced from experiments with fluorogenic peptides. Peptides 1, 3, 4, and 5 are all generated by cleavages C-terminal of a tyrosine residue, but only peptides 4 and 5 are produced in greater amounts as a consequence of REG binding. The only consistent finding is a reduction of all peptides with a leucine at their C terminus in the presence of REG. We have confirmed the impact of REG binding on cleavage preferences by using two further synthetic 25-mer peptide substrates in additional experiments (data not shown). Taken together, these *in vitro* studies suggest that binding of the REG to the 20 S proteasome contributes to the diversity in generation of antigenic peptides to a similar extent as does the incorporation of LMP subunits.

DISCUSSION

In this study, we have shown that binding of the 11 S regulator markedly alters the quality and quantity of peptide products generated by the 20 S proteasome. In the same *in vitro* digestion assay using a 25-mer peptide as a substrate, we demonstrate that single or joint incorporation of LMP2 and LMP7 subunits into the 20 S proteasome likewise changes the quantity of different petide products generated. The REG does not preferentially activate LMP2 or 7 containing proteasomes, and it does not compensate for LMP-associated alterations in proteasomal cleavage specificity. Hence, the incorporation of LMP subunits into the 20 S proteasome and binding of the REG could both function to increase the variation of peptides produced for antigen presentation on MHC class I molecules.

When the genes encoding LMP2 and LMP7 were found to map to the MHC class II region and shown to be IFN-γ inducible, the data gave an apparently congruent picture: LMP2 and LMP7 coinduction was reported to double the cleavage rate C-terminal of arginine and tyrosine in fluorogenic substrates, and it was suggested that this generates more peptides which meet the binding requirements of MHC class I molecules (38, 39). Recently, this view has been challenged by Ustrell *et al.* (40) who find no significant impact of LMP subunits on the peptidase activity of purified 20 S proteasomes or by Boes *et al.* (32) who reported that IFN-γ-mediated incorporation of LMP2 and LMP7 reduces rather than enhances the cleavage C-terminal of tyrosine residues. The data presented in Table I are in accordance with the findings of Boes *et al.* in that combined incorporation of LMP2 and LMP7 reduced the Suc-LLVY-MCA hydrolyzing activity by about 40%. The transfection approach allowed us to test if single incorporation of either LMP2 or LMP7 had any effect on the cleavage of fluorogenic peptides. This was clearly the case: incorporation of LMP2 alone, for example, reduced the cleavage of the (*Z*)-LLE-βNA by about 50%. Thus, our data obtained with fluorogenic peptides support the concept that incorporation of the LMP subunits does alter the cleavage characteristics of the 20 S proteasome. It is quite difficult to rationalize why other laboratories performing similar *in vitro* experiments obtain controversial results. We do not feel that these discrepancies can be attributed to the use of different cell line models, as we obtained identical results with

FIG. 3. **Kinetic of the degradation of a 25-mer polypeptide by 20 S proteasomes in the absence and presence of REG.** A synthetic 25-mer polypeptide (sequence displayed in Fig. 4) was subjected to digest by B8 derived 20 S proteasomes in the absence of presence of an 8-fold molar excess of REG. Aliquots of the reaction mixture were withdrawn at indicated times and separated by HPLC. The most prominent peak in the profile B8 5 h is the fragment GPSEKRVWMS generated from a cleavage C-terminal of leucine.

TABLE II

Ion current of peptide fragments produced by in vitro digest from a 25-mer polypeptide

The digests were performed as described in the legend to Fig. 4. The peptide sequences were determined by the mass/charge values (m/z) of the peptide ions detected. The relative amount of these ions specified in counts was obtained from the intensity of the corresponding ion current. It should be noted that it is not possible to compare the ion currents obtained from different peptides. The sequence of the peptides marked with asterisks was confirmed by MS/MS experiments.

Peptide sequence	m/z	B8 + REG	BC2P6 + REG	B7H6 + REG	BC27H7 + REG	B8	BC2P6	B7H6	BC27H7
						$10^3 \times$ counts			
WMS*	423.2/1	61	50	84	47	66	74	53	37
RLMYD*	697.3/1	220	92	145	131	299	0	102	144
RVWMS	678.4/2	172	117	224	0	202	37	116	125
RLMY*	582.2/1	684	1115	961	1351	896	819	997	1330
PHFMPTNL*	956.4/2	922	1085	688	720	2494	1899	1028	1567
YPHFMPTNL	1119.8/2	31	74	29	35	220	220	128	171
MYPHFMPTNL	1250.6/2	7	20	0	4	389	225	455	599
DMYPHFMPTNL*	1365.6/2	313	820	847	380	1807	2823	2973	3272
DMYPHFMP*	1037.4/2	342	353	578	317	0	0	0	0
DMYPHFM*	940.4/2	686	405	801	1077	598	327	441	635
DMYPHF	809.6/2	91	295	176	754	45	137	68	193
25-mer	3086.5/3	0	31	0	0	0	0	0	0

B8 mouse fibroblast cells, T2 lymphoblastoid cells, or Sci/ET27F pre-T cells (51), and identical results were obtained with IFN-γ stimulation or LMP2/LMP7 double transfection. A major cause of discrepancy might be the different purification protocols of 20 S proteasomes applied by different laboratories. We noticed that following our protocol a high molecular weight protease complex copurifies with the 20 S proteasome and is only separated in the last step by MonoQ chromatography. This complex is not related to the 20 S proteasome as it does not react with anti-proteasome antibodies in Western blots (data not shown), but it has protein chemical properties similar to α_1-macroglobulin-cathepsin complexes as described by Dahlmann *et al.* (52). This complex hydrolyzes fluorogenic arginine and tyrosine but not leucine substrates, and if a purification scheme does not remove this complex, the results will not be comparable to those obtained with our protocol. We performed our peptidase assays with freshly isolated proteasomes which is important because we noticed that the peptidase activity may be changed when the complex was frozen. This is a further source of controversy between different laboratories.

The sequence of our model 25-mer polypeptide is derived from the immediate early protein pp89 of the murine cytomegalovirus. It contains the nonamer YPHFMPTNL which is an immunodominant T cell epitope for the presentation on H-2L^d MHC class I molecules. In previous experiments it was not possible to directly measure the production of this nonamer *in vitro* (32). With the help of electrospray mass spectrometry, we were able to identify and quantify the production of this nonamer peptide (peptide 2 in Fig. 4): the highest amount was detected in digests conducted with proteasomes from B8 wild-type cells and the LMP2 transfectant whereas LMP7 overexpression had an adverse effect. Binding of the REG to the 20 S proteasome markedly reduced the amount of nonamer, and the reduction was least prominent in the case of the LMP2 transfectant. For the generation of this nonamer, the presence of the REG appears to be a disadvantage, whereas the production of other nonamer peptides might be favored. Apparently, the immune system varies at the level of populations by maintaining a polymorphism in MHC haplotypes with different anchor residues, at the level of single cells though, by variation in antigen processing. For this purpose the immune system may have recruited the REG. Not only binding to the proteasome itself but also the level of incorporation of either of the 29- and 31-kDa subunits into the ringshaped 11 S regulator might contribute to diversity in antigen processing. Apart from antigen presentation it might not be arbitrary which peptides are being generated during the degradation of cytosolic proteins for intracellular communication.

A puzzling question remains: how does a viral protein find an access to the interior of the 20 S proteasome? *In vitro*, the 20 S proteasome does not cleave intact proteins, with very few known exceptions, and binding of the REG apparently does not change this property of the 20 S proteasome (20, 21). Earlier data obtained with a cell line mutant, partially deficient in ubiquitin activation, had suggested that ubiquitin conjugation and degradation via the 26 S proteasome might be essential to antigen presentation (53), but as a subsequent report by other investigators has raised doubts on this pathway (54), the question remains unresolved. Theoretically, it would be conceivable that the REG binds to one α-end plate of the 20 S proteasome and a 19 S regulator complex to the opposite α-end plate, thus accepting ubiquitinated proteins. However, in the light of experimental data reported by Hoffmann and Rechsteiner (28) such a possibility seems unlikely, and further research will be required to unravel these questions.

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