

Interferon γ Stimulation Modulates the Proteolytic Activity and Cleavage Site Preference of 20S Mouse Proteasomes

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Summary

The proteasome is a 700-kD multisubunit enzyme complex with several proteolytically active sites. The enzyme complex is involved in both ubiquitin-dependent and -independent protein degradation and may contribute to the processing of antigens presented by major histocompatibility complex (MHC) class I molecules. Here we demonstrate that treatment of mouse fibroblast cells with 20 U interferon γ (IFN- γ) for 3 d induces a change in the proteasome subunit composition and that the β -type subunit LMP2, which is encoded in the MHC class II region, is incorporated into the enzyme complex. This is paralleled by reduction of the homologous δ -subunit. IFN- γ stimulation results in a downregulation of the chymotrypsin-like Suc-LIVY-MCA peptide hydrolyzing activity of 20S proteasomes whereas the trypsin-like activity remains unaffected. When tested as a substrate a synthetic 25-mer polypeptide whose sequence covers the antigenic nonapeptide YPHFMPNL of the MCMV pp89, 20S proteasomes of IFN- γ -induced cells exhibit altered chymotrypsin-like cleavage site preferences. In the absence of IFN- γ induction, the naturally processed nonamer peptide that is presented by MHC class I molecules appears as a minor cleavage product. IFN- γ activation does not result in an increase of the final peptide but results in a different set of peptides. We hypothesize that these peptides represent precursor peptides that can be trimmed to final peptide size.

The proteasome is a nonlysosomal multisubunit 700-kD multicatalytic proteinase complex that possesses at least three distinct proteolytic sites with trypsin-like, chymotrypsin-like, and peptidyl-glutamyl-peptide hydrolyzing activity (1–3). The ubiquitous enzyme complex cleaves at neutral or slightly basic pH at the carboxy side of acidic, basic, and hydrophobic residues (for review see 4). Recent work shows that there exist at least two additional proteolytically active sites, including one that is responsible for the initial cleavage of larger protein substrates degraded by 20S proteasomes (i.e., proteolytic site) (5). Since 20S proteasomes are not compartmentalized they are thought to reside in the cytoplasm in an inactive so-called latent state. In their latent conformation proteasomes are not able to cleave larger polypeptides or proteins despite the fact that the peptidyl cleavage sites are active (6).

The proteasome is a cylinder-like particle composed of four staggered rings and it exhibits a six- to sevenfold symmetry (7, 8). Thus, eukaryotic proteasomes are built up of 24–28

protein subunits with molecular masses between 35 and 21 kD. Almost 20 different proteasomal cDNAs or genes from various species have been characterized that encode related protein subunits of the α - and β -type (9, 10). This nomenclature is based on the similarity of the subunits to either the α or β subunit of the archaeobacteria proteasome (10). However, none of the subunits analyzed so far possess the sequence homologies to known proteinases.

The importance of 20S proteasomes for various intracellular metabolic pathways has become evident from gene disruption studies in yeast where all but one of an increasing number of proteasome deletion mutants revealed a lethal phenotype (9, 11). Mutational analysis also shows that the β -type subunits are involved in the generation of proteolytically active sites, whereas the α -type subunits seem to be important for assembly and the regulation of the complex (12). With regard to function, 20S proteasomes play a central role in ubiquitin-independent and, as part of a larger 26S complex, in ATP/ubiquitin-dependent intracellular protein turn-

over (1, 13, 14). There exists also increasing evidence that proteasomes are involved in mitotic events and cell proliferation (15, 16).

At least two of the proteasome genes, encoding the proteasome β -type subunits LMP2 and LMP7 map to the class II region of the MHC in close neighborhood to the peptide transporter genes (17–21). The synthesis of these proteins is inducible by IFN- γ (19, 22, 23) and both subunits exhibit haplotype-dependent variations in their electrophoretic mobility. These results, along with the fact that within a non-acidic environment 20S proteasomes cleave at the carboxy side of basic or hydrophobic residues, i.e., characteristics thought to be required for MHC class I-related protein processing (24), lead to the notion that the proteasome is the proteolytic enzyme responsible for generation of peptides presented by MHC class I molecules and this activity may implicate the function of ubiquitin-dependent proteolysis for at least some of the antigens (25).

Recently the importance of LMP2/LMP7 and of the 20S proteasome itself was questioned as it was shown that functional peptide transporter genes (TAP) are sufficient to partially restore MHC class I antigen presentation in transformed human T2 cells (26, 27). Both LMP2 and LMP7 are nonessential with regard to the basic enzymatic functions of 20S proteasome and viability of mammalian cells. Yet, their synthesis in response to IFN- γ may indicate a function of both subunits in the regulation of 20S proteasome.

So far there exists no information about the role of LMP2 and LMP7 within the proteasome complex and whether IFN- γ in fact influences proteasome activity at all. In the present communication we have analyzed the effect of IFN- γ on 20S proteasome with regard to proteasomal subunit composition, enzyme activities, and polypeptide cleavage properties as well as the ability of the proteasome to produce antigenic peptides that are presented by MHC class I molecules.

Materials and Methods

Purification and Analysis of 20S Proteasome Complexes. All chromatographic purification steps were performed at 4°C. Mouse B7 fibroblast cells were homogenized in KAc-buffer (80 mM KAc, 5 mM MgAc₂, 10 mM Hepes, pH 7.4) plus 0.1% Triton X-100 and the homogenate centrifuged at 40,000 *g* for 20 min. The resulting sol-80 supernatant (28) was incubated with equilibrated DEAE-Sephacel matrix for 45 min. Bound material was eluted with extraction buffer containing 500 mM KAc. The eluate was concentrated with a filtration system (Amicon Corp., Danvers, MA) and subjected to centrifugation on 10–40% sucrose gradients in extraction buffer. Proteasome complexes were identified by enzyme assays using the fluorogenic substrate Suc-LLVY-MCA. 20S fractions revealing peptide hydrolyzing activity were pooled and applied to fast performance liquid chromatography Mono Q column chromatography. 20S proteasome was eluted with a salt gradient between 0.1 and 1 M KCl and identified by enzyme assays as described above. Analysis of proteasome complexes by SDS-PAGE (29), two-dimensional gel electrophoresis (30) were carried out as previously described (28).

Assay of Proteolytic Activity and HPLC Separation of Processed Peptides. Purified 20S proteasome complexes were analyzed with regard to their peptide hydrolyzing activities using the fluorogenic

peptide substrates Bz-VGR-MCA HCl (trypsin-like), Suc-LLVY-MCA, and Suc-AAF-MCA (chymotrypsin-like) as described before (31). 100 ng of purified 20S proteasome were used for kinetic studies. To assay the polypeptide cleavage properties we used a synthetic 25-mer polypeptide (RLMYDM YPHFMPTNL GPSEKRVWMS) whose sequence was derived from the murine cytomegalovirus IE pp89 protein and that covers the antigenic nonamer region (32). 20 μ g of polypeptide and 1 μ g of proteasome in assay buffer (20 mM Hepes/KOH, pH 7.8, 2 mM MgAc₂, 1 mM dithiothreitol) were incubated at 37°C for 24 h. Incubations longer than 24 h had no influence on the cleavage products obtained. The total volume was 300 μ l. For analysis of the cleavage products, 50 μ l of the assay mix were applied to separation by HPLC chromatography. HPLC was performed on a reversed-phase column (Delta-Pac C18 150 \times 3.9; Waters Associates, Milford, MA) using equipment from Pharmacia LKB (Piscataway, NJ). 25 μ l of the digest was loaded and eluted with a flow rate of 1.0 ml/min in a linear acetonitrile gradient. Solution A: 20 mM ammonium acetate, pH 6.5; solution B: 50% acetonitrile and 50% solution A. 0–4 min 20% B, 4–39 min linear increase to 90% B. 0.5-ml fractions were collected and stored at –20°C or were directly subjected to the CTL assay.

Sequencing the Complex Peptide Mixture. Peptide sequencing was done by Edman degradation on an instrument by Applied Biosystems, Inc. (model 471A; Foster City, CA), 20 μ l digest was applied on the sequencer and the received data evaluated by the chromatography data system PEAKMASTER from Harley Systems (Buckinghamshire, UK).

Cell Culture. The mouse embryo fibroblast cell clone BALB.SV B7 (H-2^d) originates from the SV40-transformed cell line BALB.SV (33). Pretreatment of cells with 20 U/ml recombinant murine IFN- γ (lot No. M3-RD48; kindly provided by Dr. G. R. Adolf, Bender Vienna, Vienna, Austria) was performed for 72 h. After trypsinization, cells were intensively washed, counted, and stored at –70°C until use.

Cytolytic Assay. L^d-restricted and peptide-specific polyclonal CTL were generated by in vitro restimulation of spleen cells with the pp89-derived nonapeptide YPHFMPTNL (32) and priming of BALB/c (H-2^d) mice with the pp89 expressing recombinant vaccinia virus MCMV_{IE1}vacc. CTL were propagated by weekly peptide restimulations in the presence of recombinant human IL-2 (100 U/ml). As target cells, P815 (H-2^d) cells were labeled with Na₂⁵¹CrO₄ for 90 min. Incubation of 10³ labeled target cells/well for 1 h at 37°C with the HPLC fractions to be tested for the presence of antigenic peptide activity preceded the addition of 10⁴ effector cells/well. For the quantification of the antigenicity of the HPLC fractions, 10-fold dilution steps were performed. All fractions were tested in triplicates in a 3-h cytolytic assay.

Results

IFN- γ Induces an Exchange of Proteasome Subunits in Mouse Fibroblast Cells. The synthesis of both β -type proteasome subunits LMP2 and LMP7 is inducible by IFN- γ . This was taken as an indication that proteasomes may be involved in the cytoplasmic processing of antigens presented by MHC class I molecules (22, 23). So far our knowledge about LMP2 and LMP7 and the effect of IFN- γ on proteasome composition is solely based on immunoprecipitation of pulse-labeled proteasome subunits from unfractionated cell lysates. There exists no information on the consequences of the incorporation of these subunits into 20S proteasomes and the effect of IFN- γ

on enzyme activity of purified proteasomes under steady state conditions.

To analyze the effect of IFN- γ on the steady state protein subunit composition of the enzyme complex, purified 20S proteasomes of IFN- γ (-) and IFN- γ (+) mouse fibroblast cells were applied to analysis by two-dimensional PAGE (Fig. 1, A and B). Comparison of the resulting Coomassie-stained protein subunit patterns demonstrates that stimulation of B7 fibroblast cells with 20 U/ml of IFN- γ for 72 h leads to significant alterations in the steady state subunit composition of 20S proteasomes. Three proteasome proteins are affected predominantly. Striking is the almost complete absence of the constitutive β -type subunit δ and the concomitant incorporation of the MHC-encoded LMP2 subunit. While the LMP2 subunit is hardly detectable in proteasome complexes of nonstimulated cells, after cytokine stimulation LMP2 is incorporated into the 20S particle at an almost equal molar ratio. Both subunit δ and LMP2 exhibit amino acid sequence identity of close to 60% in humans (18, 34) and therefore may represent homologous subunits with related functional properties within the 20S multienzyme complex. In addition to subunit δ , the relative amount of a not yet characterized 27.5-kD subunit is also drastically reduced and paralleled by the appearance of a not yet characterized 27-kD protein. IFN- γ stimulation of mouse fibroblast cells also influences the posttranslational modification in that a reduction in the acidic modifications of the 32-kD MC2 α -type subunit is observed.

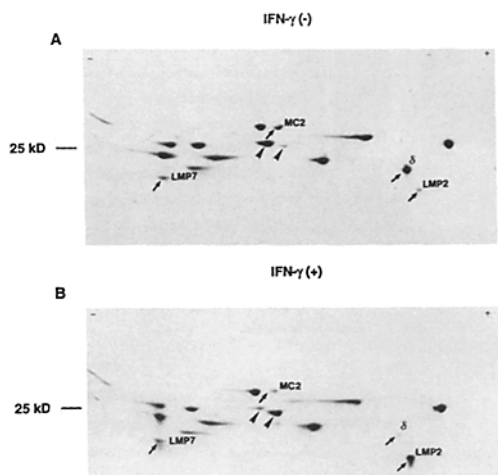


Figure 1. Effects of IFN- γ stimulation on the steady state subunit composition of purified 20S proteasomes. 20S proteasomes were biochemically purified from IFN- γ stimulated (20 U/ml) for 72 h and nonstimulated mouse fibroblast cells. The effect of the cytokine on the steady state protein composition of 20S proteasomes was monitored by two-dimensional gel electrophoretic analysis. Separated proteins were stained with Coomassie brilliant blue. (A) Protein pattern of 20S proteasomes from nonstimulated fibroblast cells. The arrows mark the subunits MC2, LMP7, δ , and LMP2; the triangular arrows mark the 27.5- and 27-kD proteins. The identity of subunits LMP2 and LMP7 was confirmed by immunoblotting with subunit specific antibodies (35). (B) Protein pattern of 20S proteasomes from stimulated fibroblast cells. Note that the δ subunit is strongly reduced while levels of LMP7 are almost unchanged.

In contrast to LMP2, the LMP7 protein subunit turns out to be a stable component of the 20S proteasome (Fig. 1, A and B, arrows). Moreover, despite the fact that the transcription of the LMP7 gene is inducible by IFN- γ (35) the relative stoichiometric quantities of this subunit in the 20S proteasome complex is apparently not significantly influenced by the cytokine.

IFN- γ Influences the Chymotrypsin-like Activity of 20S Proteasomes. Since secondary modifications or even the exchange of subunits in multienzyme complexes often result in changes of the enzymatic properties, we studied in more detail the effect of IFN- γ on the enzyme activities of the purified 20S proteasomes in vitro. The chymotrypsin- and trypsin-like activities of 20S proteasomes from IFN- γ (+) and IFN- γ (-) cells were assayed using standard fluorogenic peptide substrates. As shown in Fig. 2 A IFN- γ stimulation has no apparent effect on the trypsin-like activity of 20S proteasomes as monitored with Bz-VGR-MCA and therefore this activity could serve as an additional internal activity standard. In contrast, with the peptide substrate Suc-LLVY-MCA a dramatic 60–70% reduction in the chymotrypsin-like peptide hydrolyzing activity is observed for proteasome complexes from IFN- γ stimulated fibroblast cells (Fig. 2 B). As shown in Fig. 2 IFN- γ stimulation of fibroblast cells negatively modulates the V_{max} of the chymotrypsin-like active site(s) monitored with Suc-LLVY-MCA whereas no significant effect on the affinity for the peptide substrate as determined by K_m values is detectable (Fig. 2). The minor chymotrypsin-like activity monitored with Suc-AAF-MCA is not influenced by stimulation of the cells with the cytokine and may indicate the presence of a separate chymotrypsin-like active site (data not shown).

20S Proteasomes Reveal Altered Cleavage Site Preferences after IFN- γ Stimulation. Fluorogenic peptide substrates are suitable to characterize different proteolytically active sites of latent and active 20S proteasomes. Yet, they provide little information on how proteasomes act on larger polypeptides that are thought to represent substrates in vivo. To investigate the effect of IFN- γ on the hydrolysis of larger substrates by 20S proteasomes in vitro we used a synthetic 25-mer polypeptide whose sequence was derived from the MCMV IE pp89 protein and that harbors the antigenic nonamer peptide of IE pp89 (31). The 25-mer polypeptide is not cleaved by latent 20S proteasomes (data not shown) and thus shares in this respect characteristics with larger protein substrates. For analysis the cleavage products of the 25-mer polypeptide obtained after incubation with purified 20S proteasome complexes from IFN- γ (-) and IFN- γ (+) cells were separated by HPLC chromatography. As shown in Fig. 3 a complete turnover of the pp89 25-mer polypeptide substrate is obtained under the incubation conditions used. Furthermore, since no change in the profiles of the chromatographs was observed even after prolonged 48-h incubation periods (data not shown) we conclude that we monitor the final digestions products and that observed differences are not due to incomplete hydrolysis of the used 25-mer polypeptide substrate.

Comparison of the chromatographs demonstrates that the

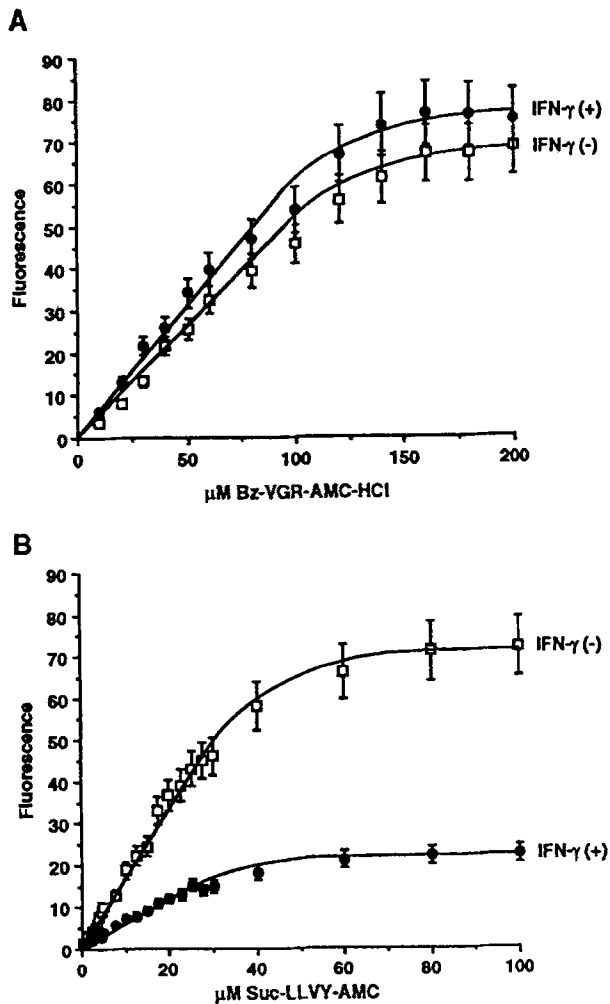


Figure 2. Influence of IFN- γ on the enzyme activity of 20S proteasomes. 20S proteasomes were purified to homogeneity from IFN- γ (-) and IFN- γ (+) mouse fibroblast cells. The enzyme kinetics for the trypsin-like and chymotrypsin-like peptide hydrolyzing activity were assayed using the fluorogenic substrates Bz-VGR-MCA (trypsin-like) and Suc-LLVY-MCA (chymotrypsin-like). (A) The kinetics of the trypsin-like activity. V_{max} values: 320 pmol/100 ng proteasome per h and 280 pmol/100 ng after IFN- γ treatment. K_m for both kinetics, 64 μ M. (B) The kinetics of the chymotrypsin-like activity. V_{max} values: 296 pmol/100 ng proteasome per h and 88 pmol/100 ng proteasome after IFN- γ treatment. K_m for both kinetics, 18 μ M. IFN- γ stimulation reduces the chymotrypsin-like activity of 20S proteasomes by ~60%. The kinetics shown are representative examples of three independent experiments.

reduction in the chymotrypsin-like activity of 20S proteasomes from IFN- γ stimulated fibroblast cells is paralleled by an alteration in the relative amounts of specific 25-mer cleavage products. In vitro digests with 20S proteasome complexes from IFN- γ (+) cells generates several pp89 25-mer cleavage products that were hardly detectable after digestion with proteasomes from IFN- γ (-) control cells (Fig. 3, A and B, arrows). This can be due to a change in the cleavage site specificity of 20S proteasomes or that IFN- γ stimulation induces a change in the preference at which 20S proteasomes use the same cleavage sites of the 25-mer polypeptide.

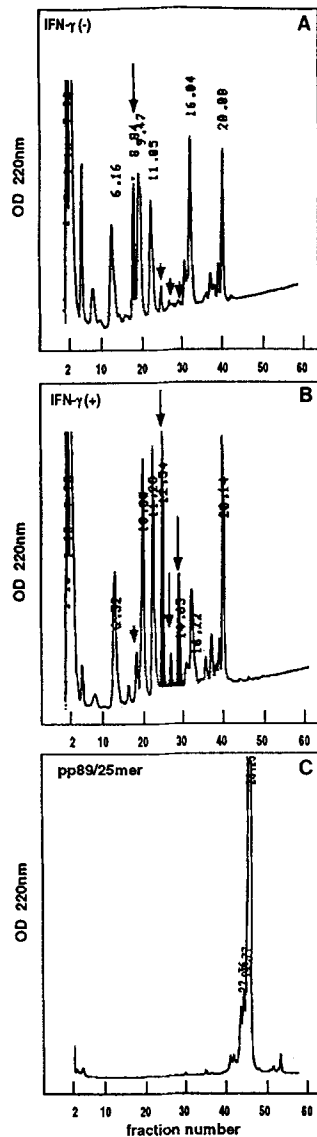


Figure 3. Analysis of the polypeptide cleavage properties of 20S proteasome from IFN- γ stimulated and nonstimulated mouse fibroblast cells. For the analysis of the polypeptide cleavage properties of 20S proteasome complexes from IFN- γ stimulated and nonstimulated fibroblast cells a synthetic 25-mer polypeptide whose sequence was derived from the pp89 IE protein of the murine cytomegalovirus was used. 20 μ g of substrate were incubated in the presence of 1 μ g proteasome at 37°C for 24 h and the cleavage products were separated by reversed HPLC chromatography. (A) Cleavage pattern obtained with 20S proteasome from nonstimulated cells. (B) Cleavage pattern obtained with 20S proteasome from IFN- γ stimulated cells. (C) Digestion of the 25-mer substrate in buffer in the absence of 20S proteasome.

To distinguish between these activities, the pp89 25-mer digests were subjected to aa sequence analysis by Edman degradation. Six important cleavage sites were observed within the 25-mer polypeptide: the trypsin-like cleavage site between K and R and the chymotrypsin-like cleavage sites behind L, Y, and V. Four of these cleavage sites that are important in this context are shown in Fig. 4. Of the cleavage sites detected those between L/M, Y/D, Y/P, and L/G were subject to alterations when proteasomes from IFN- γ stimulated cells were used (Figs. 4 and 5). As shown in Fig. 4 20S proteasome of IFN- γ (-) cells preferentially cleave the pp89 25-mer polypeptide between Y/P, a cleavage site that destroys the antigenic peptide. Remarkably, the same cleavage site is less frequently used by 20S proteasomes of IFN- γ (+) cells. In addition, proteasomes from stimulated fibroblast cells preferentially cleave between Y/D and between L/G. It is important to note that 20S proteasome complexes from both IFN- γ (-) and IFN- γ (+) fibroblast cells use the cleavage

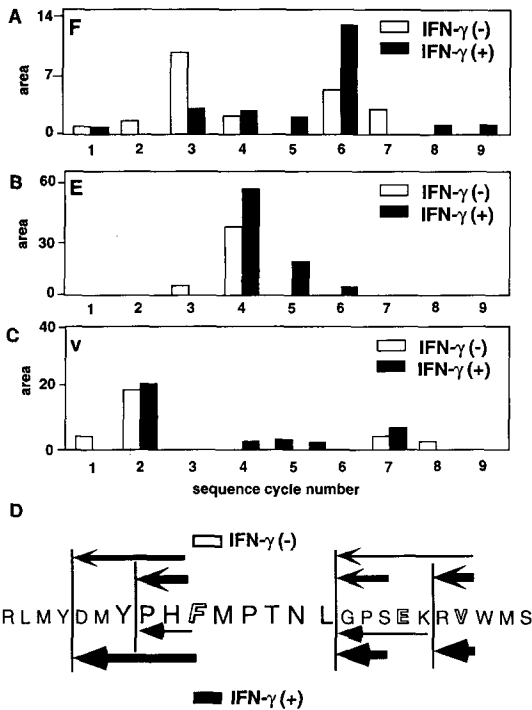


Figure 4. Modulation of the 20S proteasome by IFN- γ results in longer peptides processed from the pp89 25-mer polypeptide by altered preference for proteolytic cleavage sites. After digestion of the pp89 25-mer polypeptide by 20S proteasomes from IFN- γ (-) and IFN- γ (+) cells, the peptide mixture was applied to Edman sequencing and the summary of the evaluation of the complex chromatograms is given for the aa F, E, and V: each of them present only once in the pp89 25-mer (data summary D). (A) Proteasomes from IFN- γ (-) cells preferentially use the cleavage site between Y and P indicated by F in cycle 3. Proteasomes from IFN- γ (+) cells mainly cleave the 25-mer polypeptide between Y and D indicated by F in the cycle 6. In contrast to untreated proteasomes, longer peptides are derived from the 25-mer polypeptide because there is no major cleavage site at an earlier position, between position 5 and 10 (no strong signal of F in cycles 1–5). (B) A strong signal for E in sequence cycle 4 indicates that a major number of peptides start with G. Thus, there exists a major cleavage site between L and G that is even more effectively used by IFN- γ (+) proteasomes. (C) V detected in cycle 2 and 7 indicates a trypsin-like cleavage site between K and R and again between L and G, respectively. (D) Sequence of the pp89 25-mer polypeptide. The antigenic nonamer is indicated by capital letters. The major cleavage sites identified by Edman degradation are indicated by arrows. The thickness of the arrows reflects the relative usage of the respective cleavage sites. The relative usage of the cleavage between L/M and V/Y is not shown.

site between L and G that generates the correct carboxy-terminal residue of the antigenic pp89 nonamer at a similar frequency. In contrast, the cleavage site within the antigenic nonamer region (Y/P) is used at a strongly reduced frequency by 20S proteasomes of stimulated cells. This results in peptides that are longer at the amino terminus and that encompass the complete antigenic nonamer sequence. Thus, the cleavage specificity of 20S proteasomes is not changed, but IFN- γ stimulation alters the frequency at which specific cleavage sites of the substrate are recognized by 20S proteasome complexes. As a result, the quality of the peptide population generated is altered.

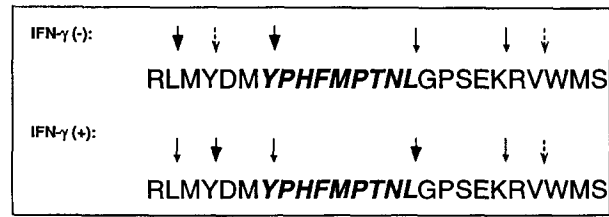


Figure 5. Cleavage site usage of 20S proteasome from IFN- γ (-) and IFN- γ (+) cells. The graph summarizes the 20S proteasome cleavage sites within the pp89 25-mer polypeptide as determined by Edman degradation. Thick arrows indicate increased cleavage frequencies.

20S Proteasome Generates a Finally Processed Nonapeptide In Vitro. To test the potential antigenicity of the cleavage products generated, pp89 25-mer polypeptide was digested in vitro by 20S proteasomes from IFN- γ (-) and IFN- γ (+) fibroblast cells. Digests were separated by reverse-phase HPLC chromatography and individual fractions were assayed with pp89-specific CTL for antigenic peptide activity. As demonstrated in Fig. 6 A, 20S proteasomes of both IFN- γ (+) and IFN- γ (-) cells produce peptides that bind to H-2L^d and induce specific CTL recognition. As a control, pp89 25-mer polypeptides incubated in assays without proteasomes contained no antigenic peptide activities. One of the peptides generated exhibits a chromatographic behavior identical with that of the synthetic nonapeptide YPHFMPTNL, which represents the naturally processed pp89 peptide (32) and coelutes in fraction 40/41 of the HPLC system used. The determination of the cleavage site usage of the pp89 25-mer polypeptide did not predict the antigenic nonapeptide as a major product. Consistently, serial dilution of fractions 40/41 revealed an $\sim 1,000$ -fold smaller amount of the nonapeptide compared with the abundant cleavage products that could be identified by sequencing and HPLC separation (Fig. 6 B). However, no significant differences were observed between digests of 20S proteasomes from IFN- γ (-) and IFN- γ (+) cells. From these data we conclude that the 20S proteasome possesses an IFN- γ independent capacity to generate the antigenic nonapeptide.

Discussion

MHC class I molecules present viral antigens as peptides at the cell surface and IFN- γ increases the number of presented peptides. Therefore, one prediction for 20S proteasome function within the MHC class I-restricted immune response is that IFN- γ stimulation of antigen-presenting cells should also affect proteasomal enzyme activity. Our analysis of mouse fibroblast 20S proteasomes demonstrates that this is indeed the case. IFN- γ stimulation of fibroblast cells results in a down-regulation of the chymotrypsin-like activity without affecting the trypsin-like activity. As shown by enzyme kinetics, this reduction in chymotrypsin-like peptide hydrolyzing activity is due to an impairment of the turnover rate (V_{max}) at the chymotrypsin-like site(s), whereas the affinity for the fluorogenic peptide substrate (K_m) appears to be unchanged.

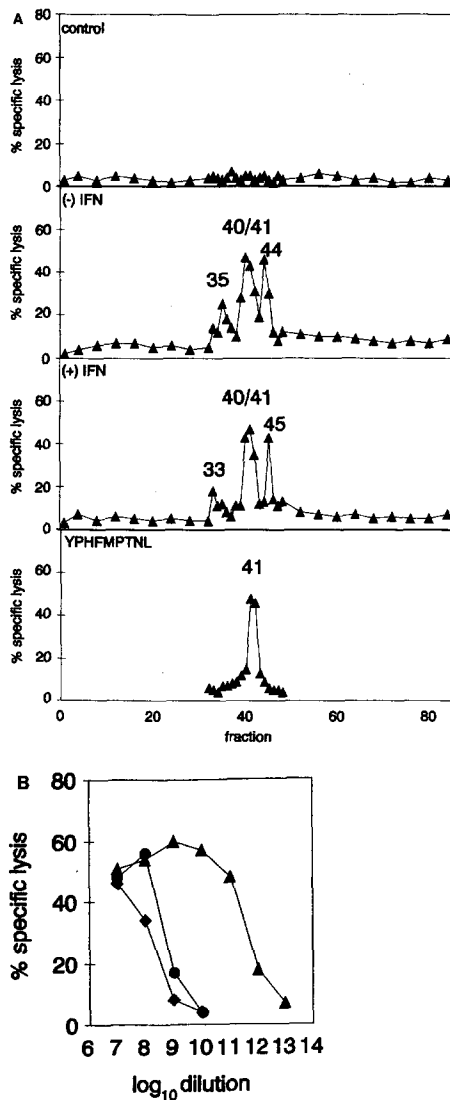


Figure 6. Processing and yield of antigenic nonapeptide by 20S proteasomes. (A) pp89 25-mer polypeptide was digested in buffer only (*control*), with 20S proteasomes prepared from either normal (*IFN- γ* (-)) or *IFN- γ* (20 U/ml for 72 h)-pretreated (*IFN- γ* (+)) fibroblast cells and cleavage products were separated by reverse-phase HPLC. Individual fractions were tested in triplicate for their content of antigenic peptide with YPHFMPTNL-specific polyclonal CTLs. Fractions with major biological activity are indicated. For comparison, the elution profile of the synthetic nonamer and the biological activity of the synthetic peptide at a concentration of 10^{-9} M is shown. (B) The biological activity that was detected in fractions 40/41 corresponding to the elution of the nonapeptide YPHFMPTNL were quantified by testing \log_{10} dilutions in the ^{51}Cr release assay. Squares indicate the titration of the respective fractions of the *IFN- γ* (+) digest, circles indicate dilution of the *IFN- γ* (-) digest. The peptide concentration of pooled fractions 40 and 41 was determined to be 1.4×10^{-7} M. An equivalent concentration of YPHFMPTNL was titrated in \log_{10} steps and tested in the cytolytic assay (*triangles*).

This cytokine dependent effect on the chymotrypsin-like activity of 20S proteasomes is paralleled by an alteration in the steady state protein subunit composition of 20S proteasomes. The most obvious change concerns the enhanced in-

corporation of the LMP2 protein to stoichiometric amounts into the 20S proteasome complex. In addition, we observed the incorporation of a not yet characterized 27-kD subunit. The same protein is also a constitutive subunit of mouse liver 20S proteasomes isolated from nonstimulated mice. Therefore, its presence is probably not the result of a primary *IFN- γ* response.

The rapid incorporation of the LMP2 subunit into the 20S enzyme complex appears to take place simultaneously with the decrease of the highly related β -type δ proteasome subunit. It is not yet proven whether this finding reflects a replacement. However, our studies using transfected human T2 cell lines that constitutively express the LMP2 subunit show that the 20S proteasomes in these cells lack the δ subunit (35a). Several β -type subunits influence the activity of chymotrypsin-like active sites (12, 36). Therefore, it is reasonable to assume that the replacement of subunit δ by LMP2 contributes to the down regulation of the chymotrypsin-like activity of the enzyme complex. Both LMP2 and subunit δ possess extensive sequence homologies to the β subunit of the thermoplasma 20S proteasome which creates the single chymotrypsin-like activity of the complex (12, 36). Finally, Wolf et al. (11) recently showed that the yeast proteasome PRE-2 subunit is homologous to LMP7 and, based on this observation, they already discussed the possibility that the constitutive δ subunit may be replaced by LMP2 upon *IFN- γ* induction.

IFN- γ does not significantly influence the half life of 20S proteasomes (35a). Therefore, the rapid incorporation of equimolar amounts of LMP2 protein into 20S proteasomes and the replacement of the δ subunit must, to a large extent, take place in preexisting cytosolic proteasomes and can not be explained by de novo synthesis of proteasomes. Alterations in proteasome subunit composition have been observed before during development (37). Therefore, the substitution of subunits may represent a mechanism to adapt proteasome function to changing physiological requirements.

In contrast to LMP2, the related LMP7 subunit is a constitutive component of B7 fibroblast 20S proteasomes and its incorporation into the complex is not significantly influenced by *IFN- γ* . The constitutive presence of LMP7 may be cell line specific since it is not observed in human cell lines where the incorporation of both subunits is strongly enhanced after *IFN- γ* stimulation (22, 23).

IFN- γ mediates the reduction of the chymotrypsin-like peptide hydrolyzing activity as determined by fluorogenic peptide substrates. The longer peptide substrate (25 mer) demonstrates a change in the frequency at which specific cleavage sites within the MCMV pp89 25-mer polypeptide are used. This divergence of cleavage activity detected by long and short substrates indicates to us that peptide configuration may contribute to proteasome function. In this context it should be recollected that the latent proteasome, i.e., proteasomes that possess peptide hydrolyzing activity but can not cleave larger substrates, cleaves the short peptides but not at all the longer 25-mer polypeptide. Thus, both assays detect different qualities of the proteasome. *IFN- γ* activation results in the production of a different set of peptides. However, it is important

to emphasize that IFN- γ stimulation does not generate new cleavage site specificities and thus the intrinsic enzymatic properties of the enzyme complex remain preserved.

The 25-mer polypeptide delineated from the IE pp89 of MCMV used in these studies contains the nonapeptide sequence YPHFMPTNL which represents the naturally processed dominant antigenic peptide that is presented by the MHC class I molecule L^d. L^d-restricted CTLs with specificity for this peptide were used to monitor the generation of biological activity. Its identity with the synthetic nonapeptide was tested by the elution profile of the activity in the HPLC. The *in vitro* studies indicate that both preparations, 20S proteasomes from normal and from IFN- γ -induced cells, are able to generate a minor biological activity that is indistinguishable from the synthetic peptide. If such peptides get access to transporter functions they provide a basic level of peptide presentation that is consistent with the observation that antigen presentation by a cell lacking both the TAP and the LMP genes can be achieved by reconstitution of only the TAP genes (26, 27).

The LMP genes are inducible by IFN- γ and if they contribute to antigen processing, the cleavage products from 20S proteasomes of IFN- γ -induced cells should show this potential. Our observation that proteasomes from IFN- γ (+) cells did not produce more biological activity *in vitro*, as detectable by CTLs, demonstrates that IFN- γ does not activate the proteasome directly to produce the final peptide. However, proteasome complexes from IFN- γ (+) cell produce a qualitatively different set of peptides.

These conclusions are based on the following result. Already in the absence of IFN- γ , but even more so after IFN- γ induction, the carboxy-terminal leucine residue of the nonapeptide represents a major cleavage site. To cleave after basic or hydrophobic residues is a general functional property of 20S proteasomes (see also above). Therefore, the precise cleavage behind such residues could provide a transport signal and contribute to the observation that many carboxy-terminal residues in naturally found antigenic peptides share this property (38).

Testing of short substrates reveals an overall decrease of the chymotrypsin-like activity after IFN- γ induction. Testing of the 25-mer polypeptide provides the additional information of a selective and differential usage of the chymotrypsin-

like cleavage sites. Without IFN- γ the position between Tyr and Pro represented a major cleavage signal that is associated with a loss of the amino-terminal Y of the nonapeptide, and in consequence with the loss of antigenic activity of the resulting peptides (32, 39). 20S proteasomes from IFN- γ -treated cells cleave at a more distant site leaving the nonamer intact. This 11-mer polypeptide is generated *in vitro* as a major fraction sufficient for direct isolation and sequencing (data not shown). Yet, without further amino-terminal trimming this 11-mer polypeptide has little activity (32). Thus, the reduction of cleavage sites results in the generation of longer peptides that have the potential to be converted to biologically active shorter peptides. Whether this represents a general principle has to be shown using additional polypeptide substrates.

Based on our data we hypothesize that it is the IFN- γ -dependent function of the LMP genes to contribute to the generation of different and larger pools of precursor peptides. Accordingly, the loss of one or both genes would not destroy peptide presentation but reduce the potential diversity of the peptide pool. The generation of longer peptides requires further degradation in the cytosol or the endoplasmic reticulum. If, as in the example tested here, only amino-terminal trimming is required, the trimming could occur even at the MHC I molecule itself, as has been suggested earlier (38).

During revision of this manuscript two papers on related topics have been published (40, 41). In essence, these papers also describe a difference in peptide cleavage activity of short fluorogenic substrates by IFN- γ -activated proteasomes. Differently from us, both groups report on an increase in the chymotrypsin-like and trypsin-like activity. First, in contrast to the fibroblast used in this study, these authors studied hepatoma cells and lymphoid cell lines. Second, the IFN- γ induction protocols of 1,000 U/ml for 3 d (41) or 20 U/ml for 14 d (40), respectively, differ significantly from our protocol of 20 U/ml for 3 d that was aimed to reflect more closely the conditions found *in vivo*. In this context it is worth noting that the more vigorous induction protocol (41) also led to a similar increase in enzyme activity even in the MHC-deficient 712.174 cells that lack LMP2 and LMP7 altogether. Finally, in agreement to our polypeptide data, the latter authors also found that cleavage of fluorogenic substrates does not reflect the activity on protein substrates. The effect on larger peptides and proteins was not further studied.

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