Newly Identified Pair of Proteasomal Subunits Regulated Reciprocally by Interferon γ

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Summary

Interferon (IFN) γ induces replacements of the proteasomal subunits X and Y by LMP7 and LMP2, respectively, resulting in an alteration of the proteolytic specificity. We found a third pair of proteasome subunits expressed reciprocally in response to IFN-γ. Molecular cloning of a cDNA encoding one subunit designated as Z, downregulated by IFN-γ, showed that it is a novel proteasomal subunit with high homology to MECL1, which is markedly induced by IFN-γ. Thus, IFN-γ induces subunit replacements of not only X and Y by LMP7 and LMP2, respectively, but also of Z by MECL1, producing proteasomes responsible for immunological processing of endogenous antigens. When processed from their precursors, three pairs of the 10 homologous, but distinct, β-type subunits of eukaryotic proteasomes, that is, X/LMP7, Y/LMP2, and Z/MECL1, have an NH_2-terminal threonine residue, assumed to be part of a catalytic center. These findings suggest that the altered molecular organization of the proteasome induced by IFN-γ may be responsible for acquisition of its functional change.

Proteasomes are present in cells as both 20S and 26S isoforms with molecular masses of ∼750 and 2,000 kD, respectively (for reviews see references 1–3). The eukaryotic 20S proteasome is a cylindrical particle consisting of four rings, each of which is organized from seven homologous, but not identical, α and β subunits, assembled in the order αββα (4). All proteasomal genes examined so far encode previously unidentified proteins, which show considerable intersubunit homology in the same species and high evolutionary conservation in various eukaryotes (5). The 26S proteasome appears to be a dumbbell-shaped particle with two large terminal subsets attached in opposite orientations to a smaller, four-layered central 20S proteasome (6, 7).

The 20S proteasome is a multicatalytic proteinase responsible for endoproteolytic cleavage of peptide bonds on the carboxyl side of acidic, basic, and hydrophobic amino acid residues of proteins (8). The manifestation of multiple catalytic activities at independent active sites within a single enzyme complex appears advantageous for the rapid and complete breakdown of various types of cellular proteins. The multicatalytic functions of proteasomes may also generate suitable types of peptides from target proteins, as proteasomes have recently been implicated in processing for

MHC class I–restricted antigen presentation (see below). Although the 20S proteasome is able to hydrolyze various small peptides, it is not responsible for endoproteolytic breakdown of any kind of large protein substrates. In contrast, the 26S proteasome is a eukaryotic ATP-dependent protease degrading a variety of cellular proteins that have a specific degradation signal(s) such as a multiubiquitin chain (9, 10). Covalent attachment of ubiquitin to target proteins acts as a degradation signal for their selective breakdown (11). The ubiquitin–proteasomal pathway appears to be involved in the selective removal of regulatory proteins with a rapid turnover related to cell cycle progression, transcription, and metabolic regulation (12, 13). In addition, the 26S proteasome is also responsible for ATP-dependent degradation of ornithine decarboxylase, which has an extremely rapid turnover rate. The degradation does not depend on ubiquitination but on the association of ornithine decarboxylase with its specific inhibitor protein antizyme (14). Intriguingly, the ubiquitin–proteasome system catalyzes not only exhaustive proteolysis of these naturally occurring short-lived proteins and abnormal/malfolded proteins generated in cells (15). It also activates the transcriptional factor NF-κB through conversion of the p105 precursor to p50

mature protein by limited proteolysis (16). Thus the proteasome is a most important enzyme responsible for the energy-dependent nonlysosomal proteolytic pathway.

The ubiquitin–proteasome system has also been shown to be involved in the processing of endogenous antigens, which are presented with the class I cell surface MHC, because marked suppression of the generation of antigenic peptides was observed both in cells with a temperature-sensitive ubiquitin-activating enzyme (E1) at the restrictive temperature (17) and in cells where proteasome function was suppressed with peptide–aldehyde inhibitors (15). Thus the proteasome is implicated to be an enzyme responsible for the processing of intracellular antigens to generate peptides, which are then transported into the endoplasmic reticulum through the transporter associated with antigen processing (TAP)[1] peptide transport system (for review see reference 18).

Studies on the effect of IFN-γ are of particular interest for revealing the exact role of the proteasome in the immune system, because IFN-γ is a major immunomodulatory cytokine. Actually, IFN-γ alters the proteolytic specificity of proteasomes, modulating their ability to generate peptides for antigen presentation (19–21). Two polymorphic MHC-encoded proteasomal genes, LMP2 and LMP7, were found to be localized in the class II MHC region closely linked to two homologous genes, TAP1 and TAP2, which are responsible for transport of peptides from the cytosol into the endoplasmic reticulum (22). The involvement of LMP2 and LMP7 in the processing pathway has been demonstrated directly by gene disruption in mice (23, 24).

Recently, we found that IFN-γ induced not only marked synthesis of the MHC-encoded proteasome subunits LMP2 and LMP7, but also almost complete loss of two unidentified proteasome subunits designated as X and Y in cells (21). Similar results have also been presented by other investigators (25, 26). Molecular cloning of cDNAs encoding X and Y showed that X (also named MB1 or ε) and Y (equivalent to δ) are novel proteasomal subunits with high homology to LMP7 and LMP2, respectively (27–29). This adaptation is proposed to be due to IFN-γ–dependent subunit replacements of X and Y by the structurally similar subunits LMP7 and LMP2, respectively, because these pairs of subunits with extremely high amino acid similarity have been shown to be expressed reciprocally in response to IFN-γ (30). However, the necessities of LMP2 and LMP7 in the antigen processing pathway are controversial, because their deletions were found to have no significant effect on the presentation of endogenous antigens (31–34).

In this study, we examined the role of IFN-γ in more detail and found two new subunits regulated reciprocally in response to IFN-γ. Molecular cloning of a cDNA-encoding subunit Z, downregulated by IFN-γ, showed that this subunit has high homology to MECL1, which is markedly

upregulated in response to IFN-γ. This is the first indication that IFN-γ induces subunit replacements of not only X and Y by LMP7 and LMP2, respectively, but also of Z by MECL1, producing proteasomes that would modulate processing of endogenous antigens. These findings are of considerable importance in clarifying the role of the proteasome in the antigen-processing pathway.

Materials and Methods

cDNA Cloning. A cDNA library of human hepatoblastoma HepG2 cells was constructed in a ZAPII phage expression vector (Stratagene Inc., La Jolla, CA). For isolation of cDNA for subunit Z, ~5 × 10^5 plaques were screened by hybridization with a cDNA fragment that had been synthesized by PCR and labeled with [α-32P]dCTP. After plaque hybridization, the pBluescript plasmid was excised and directly sequenced by a double-strand strategy in an automated DNA sequencer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Chromosomal Mapping. Direct R-banding fluorescence in situ hybridization (FISH) based on FISH combined with the replicated prometaphase R-band was applied. For suppression of repetitive sequences contained in this clone, we used human Cot-1 DNA (Bethesda Research Laboratories, Gaithersburg, MD) as described by Lichter et al. (35) with slight modifications (36). Labeling, hybridization, rinsing, and detection were carried out by routine procedures. Provia 100 (ISO100; Fuji, Tokyo) was used for microphotography (filter combination, B-2A; Nikon, Tokyo). We isolated four independent genomic clones of proteasome Z (Z-3, -5, -12, and -13) and used each clone as a probe. These clones contained an insert of ~40 kb in the pWE15 cosmid vector. The library was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

Immunological Analyses. 50-μg samples of cell extracts were subjected to SDS-PAGE and then used for immunoblot analysis (21). mAbs MCP168 and MCP421 (37) against Z and Y, respectively, and polyclonal antipeptide antibodies against subunits LMP2, LMP7, X, and MECL1 were used.

Northern Blot Analysis. 10-μg samples of total RNA were separated by electrophoresis in agarose gel containing formaldehyde, transferred to a nylon membrane (Hybond-N+; Amersham Corp., Arlington Heights, IL) and hybridized with 32P-labeled probes as described previously (21). For Northern blot analysis of proteasomes, cDNAs for subunits LMP2, LMP7 (21), X, and Y (28) of human proteasomes and the cDNA of β-actin (Oncor Inc., Gaithersburg, MD) were used. Approximately 820-bp cDNAs of MECL1 of human proteasomes were synthesized by PCR based on reported sequences (38). These probes were labeled with a commercial kit for multiprime DNA labeling (Takara Shuzo, Kyoto, Japan). The membranes were washed and autoradiographed with XAR-5 film (Eastman Kodak Co., Rochester, NY) at −70°C using an intensifying screen.

Computer Analysis. Computer-assisted homology research was carried out using the databases listed in LASL-GDB (GenBank) and NBRF-PDB.

Results

IFN-γ–dependent Changes in Subunit Composition of the Proteasome. The effect of IFN-γ on the functions of the proteasome is quite controversial (19–21, 25, 39). To clar-

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[1]*Abbreviations used in this paper:* 2D-PAGE, two-dimensional PAGE; TAP, transporter associated with antigen processing.

ify, therefore, the exact role of IFN-γ on the structure of the proteasome, we first reinvestigated carefully whether IFN-γ alters its subunit pattern in human cells. HeLa cells were metabolically labeled with [³H]leucine, and their proteasomes were immunoprecipitated and subjected to two-dimensional PAGE (2D-PAGE). Spots corresponding to LMP2 and LMP7, induced by IFN-γ, and subunits X and Y, downregulated by IFN-γ (Fig. 1), were observed, as reported previously (25–27). On closer inspection of the fluorogram, we found two other spots of subunits whose expressions are controlled reciprocally by IFN-γ (Z and MECL1, Fig. 1 A and B). Similar alterations of unidentified subunits were also found in mouse cells (25), although it is unknown whether these subunits correspond to those of HeLa cells.

To characterize these two proteins, we immunoprecipitated proteasomes from extracts of HeLa cells with or without IFN-γ treatment and separated them in larger amounts

by 2D-PAGE and blotting. The NH₂-terminal sequence of the protein upregulated by IFN-γ (circled in Fig. 1 D) was determined by Edman degradation directly from the blot. The sequence, XTIAGLVFQDGVILGADTR, is identical to that of MECL1, which has been cloned as a unique gene within a tight cluster of five unrelated human genes on chromosome 16q22.1 (38). The protein downregulated by IFN-γ (circled in Fig. 1 C) has been shown to react with mAb MCP168 (37) and has been named subunit Z. Sequencing of a proteolytic fragment of Z (ITPLEIEVLEEX-VQTMD) showed that Z is a novel proteasomal subunit.

High Similarity of the Proteasomal Subunit Z Downregulated by IFN-γ to MECL1 Expressed Reciprocally. Full-length cDNA encoding Z was then isolated by screening a human HepG2 cell cDNA library by hybridization with a cDNA fragment synthesized by PCR based on the amino acid sequence of this fragment. The nucleotide sequence of the Z cDNA clone and the primary structure of the Z protein de-
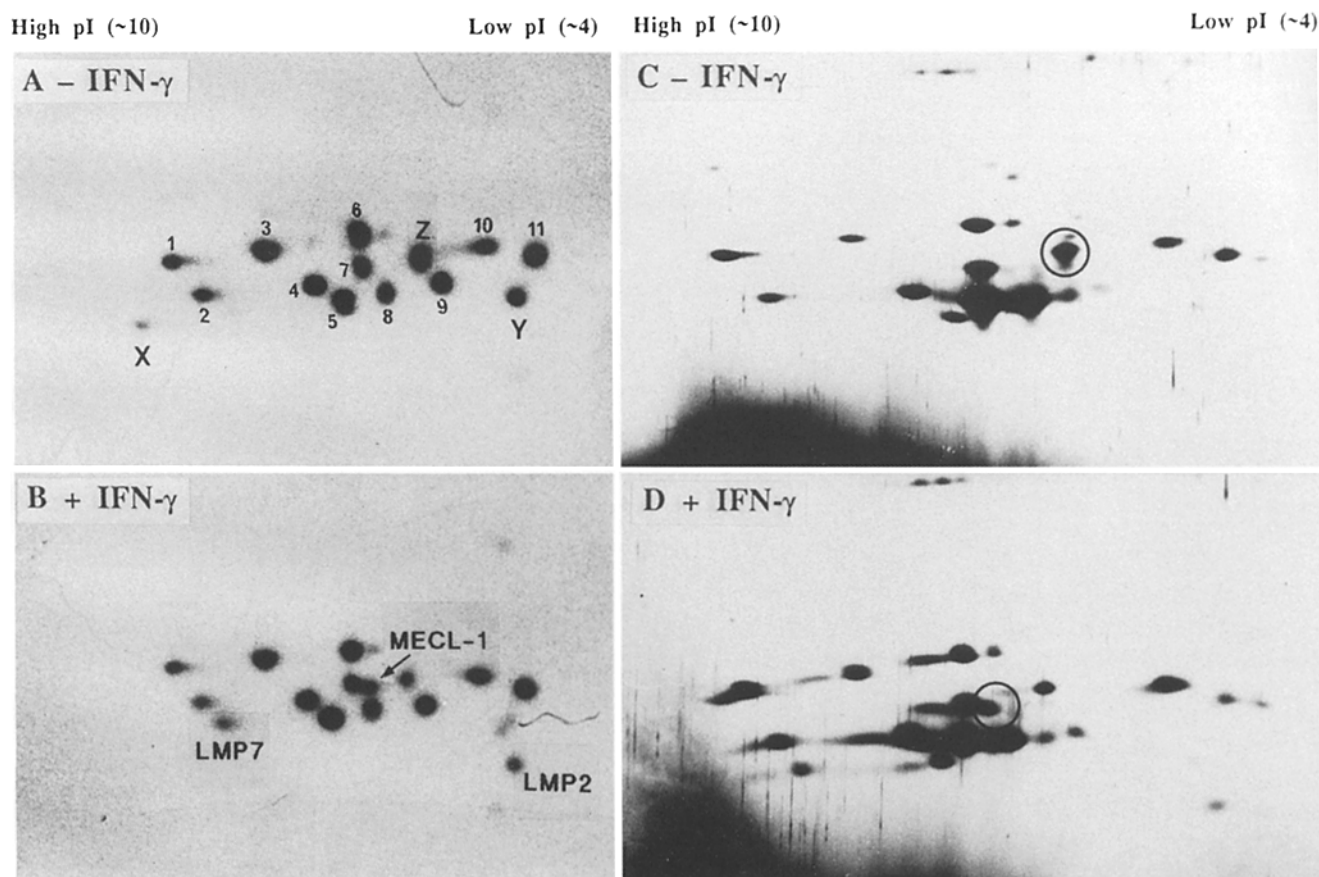

Figure 1. Identification of human proteasomal pair subunits Z and MECL1 reciprocally regulated by IFN-γ. (*A* and *B*) Effect of IFN-γ on the subunit pattern of newly synthesized proteasomes in HeLa cells. Cells were labeled for 3 d with [³H]leucine in the absence or presence of IFN-γ. Cell extracts were then treated with antiproteasomal antibodies, and the resulting immunoprecipitates were examined by 2D-PAGE and fluorography (21). The spots of MHC-encoded proteasome subunits LMP2 and LMP7 and the two spots of X and Y were up- and downregulated, respectively, by IFN-γ treatment. In addition to these four spots, the intensities of two other spots were changed reciprocally by IFN-γ treatment. Other proteasomal subunits were identified by protein sequencing and immunoblot analysis as described (37) and are numbered as follows: 1, XAPC7; 2, C5; 3, C9; 4, C3; 5, C7; 6, C2; 7, Iota; 8, C10; 9, N3; 10, C8, and 11, Zeta. Proteasomes were immunoprecipitated with antiproteasomal antibodies from unlabeled HeLa cells with or without IFN-γ treatment, separated by 2D-PAGE, and stained with Coomassie brilliant blue (*C* and *D*). The newly identified spots that increased and decreased on IFN-γ treatment are circled. On protein sequencing by automated Edman degradation, the spot upregulated by IFN-γ was identified as MECL1, and the spot downregulated by IFN-γ was a newly identified subunit tentatively designated as Z (see text).

duced from the cDNA sequence are shown in Fig. 2. The sequence of 980 nucleotides included the entire coding region and 5'- and 3'-noncoding regions. A putative polyadenylation signal (AATAAA), which is common to eukaryotic mRNAs, was located 26 nucleotides upstream from the poly(A) addition site. We concluded that ATG, located at nucleotides 1 to 3, is the initiation codon, because it is surrounded by a sequence that is similar to the consensus sequence for translation initiation (40), and because the open reading frame from this ATC ; the longest. The amino acid sequence shown in Fig. 2 was confirmed to be that of Z of proteasomes by the fact that a sequence of the fragment determined chemically (residues shown by a continuous line in Fig. 2) closely matched. Subunit Z consists of 277 amino acid residues with a calculated molecular weight of 29,965. Its isoelectric point was calculated to be 7.61 (41).
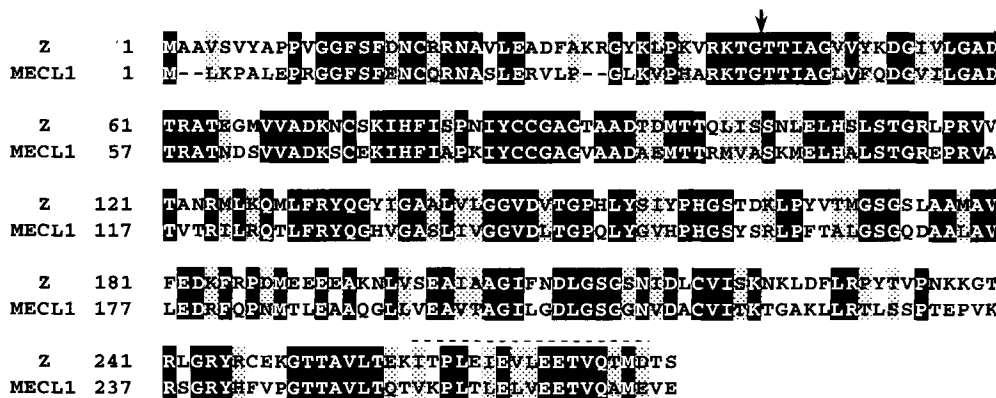
Proteasome subunits have high intersubunit similarities but can be classified into two subfamilies with high similarities to the α and β subunit, respectively, of the archaebacterial proteasome (42). Computer-assisted homology analysis showed that subunit Z belongs to the β-type proteasome subfamily, which is related to expression of the catalytic functions of proteasomes (4). Moreover, β-type subunit Z was found to be remarkable similar to proteasome subunit MECL1 (58.3% identity, excluding the NH$_2$-terminal region; see Fig. 3). This fact and the observation that the expressions of Z and MECL1 were regulated reciprocally in response to IFN-γ (Fig. 1, A and B) suggest that subunit Z can be replaced by MECL1 in the proteasomal complex, just as X and Y can be replaced by LMP7 and LMP2, respectively (28, 29).

Chromosome Mapping. To determine the chromosomal location of the gene encoding Z, we examined 100 typical R-band plates of each clone using four independent clones. The signals with the four probes were localized to the same map position. In the plate probed with clone Z-13, 43% exhibited complete twin spots on both homologues, 46% gave incomplete single and/or twin spots on either or both homologues, and the others (11%) gave no detectable spots. The signals were localized to the q34.11-q34.12 band of chromosome 9. No doublet signals were observed on other chromosomes (Fig. 4). This location clearly differs from that of MECL1 (16q22.1) (38).

IFN-γ–inducible Replacements of Three Pairs of Proteasomal Subunits with Possible Catalytic Functions. Subunit Z may be synthesized in a precursor form, a general feature of β-type subunits of proteasomes. The NH$_2$-terminal structure of a proteasomal subunit (named α, [44]TTIAGVVYKDGIVLG-ADT[61], shaded residues in Fig. 2) was found by Edman degradation (43, 44) to be identical to the internal sequence of the largest open reading frame of the Z gene. Interestingly, MECL1 seems to be processed to the mature protein at a similar site to Z, because we determined the NH$_2$-terminal sequence of MECL1 to be [40]TTIAGLVFQDGVIL-GADTR– – – – – (see arrow in Fig. 3). The isoelectric points of the mature Z and MECL1 proteins were calculated to be 5.54 and 6.07, respectively, and their molecular
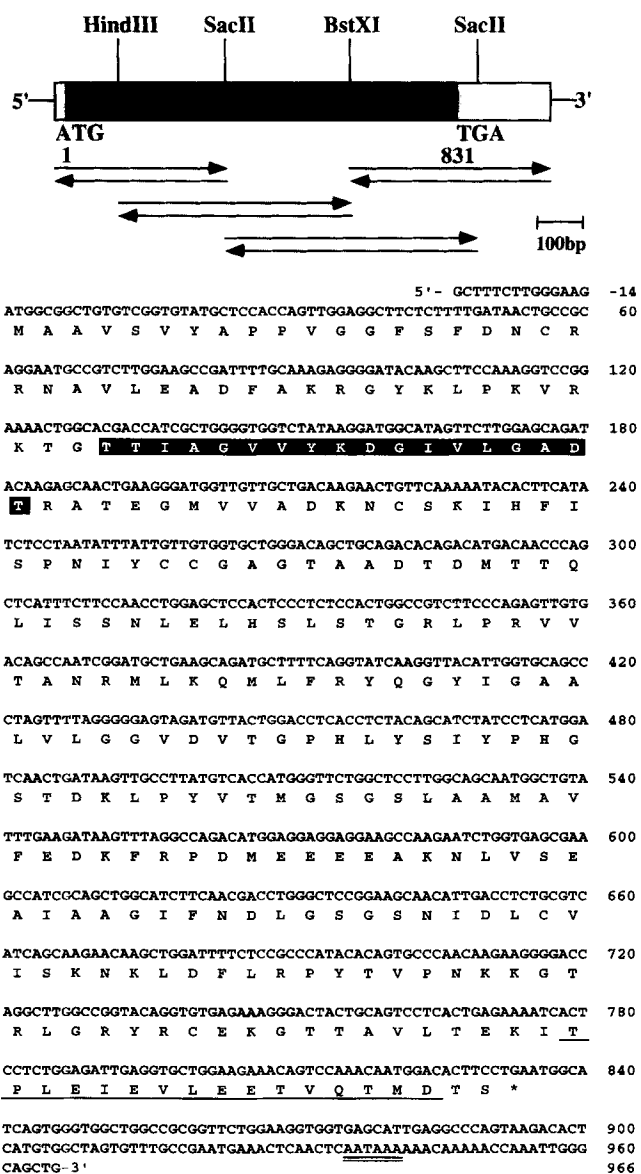
Figure 2. Structure of cDNA for subunit Z of human proteasomes. (*Top*) Restriction endonuclease map of cloned cDNA for Z and sequencing strategy. The solid and open boxes show the coding region and 5'- and 3'-noncoding regions, respectively. Continuous lines indicate the sequence of the vector, Bluescript I KS$^+$. The numbers below the boxes indicate the nucleotide numbers of the first nucleotide of the initiation codon, ATG, and the nucleotide before the termination codon, TGA. Sequenced regions are shown by horizontal arrows. The bar represents 100 bp. (*Bottom*) Nucleotide sequence of the cDNA encoding component Z and the amino acid sequence deduced from its open reading frame. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue of the putative initiation methionine codon [1]ATG[3]. The nucleotides on the 5' side are indicated by negative numbers. The predicted amino acid sequence of Z is shown below the nucleotide sequence. Amino acid residues are numbered from the NH$_2$ terminus. The amino acid sequence corresponding to that obtained by Edman degradation of a fragment cleaved with lysylendopeptidase is underlined. The sequence of the NH$_2$-terminal region, shown by shaded boxes, has been sequenced randomly by Edman degradation (43, 44). The termination codon TAA is marked with an asterisk. The possible polyadenylation signal (AATAAA) is double underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number D38048.

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Z        1   MAAVSVYAPPVGGFSFDNCRRNAVLEADFAKRGYKLPKVRKTGTTIAGVVYKDGIVLGAD
MECL1    1   M--LKPALEPRGGFSFNCQRNASLERVLP--GLKVPHARKTGTTIAGLVFQDCVLGAD

Z       61   TRATEGMVVADKNCSKIHFISPNIYCCGAGTAADTDMTTQLISSNLELHSLSTGRLPRVV
MECL1   57   TRATNDSVVADKSCEKIHFIAPKIYCCGAGVAADAEMTTRMVASKMELHALSTGREPRVA

Z      121   TANRMLKQMLFRYQGYIGAALVLGGVDVTGPHLYSIYPHGSTDKLPYVTMGSGSLAAMAV
MECL1  117   TVTRILRQTLFRYQGHVGASLIVGGVDLTGPQLYGVHPHGSYSRLPFTALGSGQDAALAV

Z      181   FEDKFRPDMEEEEAKNLVSEAIAAGIFNDLGSGSNIDLCVISKNKLDFLRPYTVPNKKGT
MECL1  177   LEDRFQPNMTLEAAQGLLVEAVTAGILGDLGSGGNVDACVITKTGAKLLRTLSSPTEPVK

Z      241   RLGRYRCEKGTTAVLTEKITTPLEIEVLEETVQTMDTS
MECL1  237   RSGRYHFVPGTTAVLTQTVKPLTLELVEETVQAMEVE
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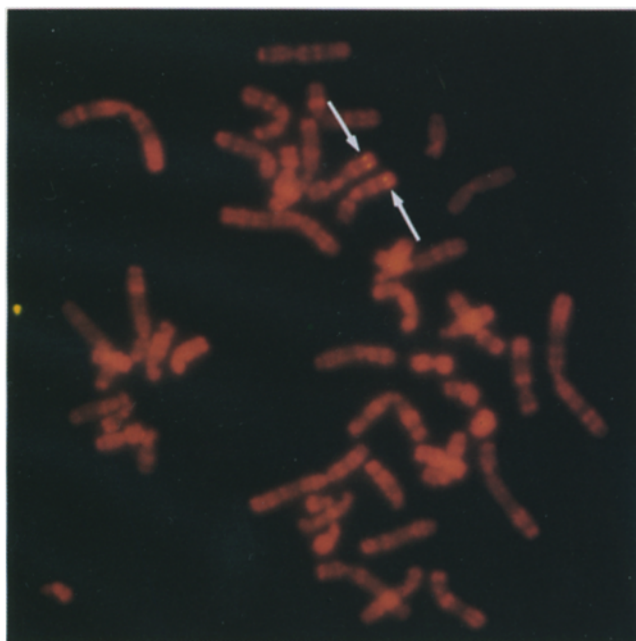
weights 25,295 and 24,648, which are roughly consistent with the positions of Z and MECL1 on 2D-PAGE (Fig. 1).

Recently the NH₂-terminal threonine residue of the *Thermoplasma acidophilum* proteasome β subunit processed from the precursor was found to be essential for the proteolytic function, indicating that the proteasome is a threonine protease with a different catalytic mechanism from those of other proteases known so far (45). This assumption is consistent with a recent observation that the novel proteasome-specific microbial inhibitor lactacystin modifies the NH₂-terminal threonine residue of subunit X and possibly Z specifically and covalently, resulting in almost complete inhibition of proteolytic activity (46). Several eubac-

Figure 4. Partial R-banded metaphase plates after in situ hybridization with a fluorescent Z DNA probe. Arrows indicate the signals on 9q34.11-q34.12 for chromosomal localization of the human proteasomal Z gene.

terial putative β subunits, *Escherichia coli* HslV and *Rhodococcus* β1 and β2 gene products (47), have the same NH₂-terminal structure (for review see reference 48). It is therefore of particular interest that the NH₂ termini of Z and MECL1 are two sequential threonine residues (TT- -) and that the IFN-γ-induced subunits X and Y and the counterparts they replace, LMP7 and LMP2, respectively, also have two NH₂-terminal threonine residues (28). In contrast, the other four human β-type subunits, HC5, HC7, HC10, and HN3, do not, suggesting that the latter four subunits may be inactive (45). So, all the active subunits with two NH₂-terminal threonine residues seem to be regulated by IFN-γ, which perhaps accounts for the changes in the proteolytic functions of the proteasome.

So far, all 14 (7α and 7β) proteasomal genes of *Saccharomyces cerevisiae* have been isolated (49). One of them, named PUP1, which is an essential gene (50), was found to show high similarities with Z and MECL1. The similarity of Z to PUP1 is ~54.8%, which is higher than the 46.5% similarity of MECL1 to PUP1. This suggests that the actual homologue of PUP1 may be Z, rather than MECL1, and that the MECL1 gene may have been generated from the Z gene by gene duplication. Fig. 5 shows results of statistical analyses of the similarities of human proteasomal subunits. The presence of 10 β subunits is curious considering that one β-type ring is composed of seven subunits. However, from the present and previous studies (28, 29), three β-type subunits, X, Y, and Z, appear to be replaced by very homologous, but different, gene products, LMP7, LMP2, and MECL1, respectively, in response to extracellular signals, such as IFN-γ.

Effect of IFN-γ on the Expressions of Three Pairs of Homologous Subunits of Proteasomes. We compared the expressions of Z and MECL1 with those of other genes of proteasomal subunits responding to IFN-γ. IFN-γ greatly increased the level of the mRNA encoding MECL1 in renal carcinoma KPK-1 and ACHN cells (Fig. 6) and in other human cells such as colon carcinoma SW620 and monocytic leukemia J111 cells (data not shown). Similar increases of the mRNAs

|	HC2	HC3	HC8	HC9	XAPC7	PROS-2	Zeta	Z	MECL1	X	LMP7E1	LMP7E2	Y	LMP2	HC5	HC7-I	HC10-I	HN3
α- Type Subunits																		
HC2	100.0	30.7	22.6	28.0	32.8	23.1	28.9	18.2	18.8	17.5	15.8	15.8	15.3	14.3	13.1	15.7	16.6	16.1
HC3	29.4	100.0	28.1	38.1	37.2	34.8	32.0	15.9	18.8	23.3	19.2	19.2	15.9	14.3	16.6	17.9	15.2	15.0
HC8	23.2	30.0	100.0	25.0	29.3	28.8	31.9	16.4	15.2	13.5	11.2	11.2	12.4	15.2	12.5	12.6	11.1	15.6
HC9	27.2	35.8	26.2	100.0	34.2	26.6	33.0	19.0	24.6	21.1	18.3	18.3	16.7	16.8	22.0	17.6	16.2	17.0
XAPC7	32.2	36.2	29.7	35.0	100.0	31.9	35.0	22.9	24.0	17.6	17.6	17.6	20.6	20.7	15.7	16.2	16.3	15.0
PROS-2	22.7	33.6	29.3	31.0	31.9	100.0	23.1	19.8	19.2	16.9	15.7	15.7	16.3	19.2	19.2	18.3	15.1	14.4
Zeta	29.7	30.9	31.9	34.8	37.5	25.1	100.0	22.0	24.9	19.7	20.2	20.2	22.6	21.6	20.6	16.1	16.4	16.8
β- Type Subunits																		
Z	16.9	15.4	14.1	15.8	20.0	16.9	18.6	100.0	62.6	27.9	27.4	27.4	33.0	29.8	19.7	20.4	20.1	21.0
MECL1	15.8	16.8	12.2	20.2	19.8	17.0	22.3	57.1	100.0	27.9	29.0	29.0	30.2	30.3	18.8	21.5	17.2	19.9
X	17.2	21.0	13.7	19.8	16.8	14.7	18.9	25.6	26.6	100.0	72.2	72.2	32.4	30.3	17.5	20.2	21.2	24.9
LMP7E1	14.6	19.2	11.4	16.2	15.8	14.4	17.2	21.1	23.1	68.3	100.0	100.0	32.4	33.7	15.8	22.0	20.0	28.8
LMP7E2	14.6	18.8	11.0	15.7	15.8	14.0	16.8	21.1	23.1	68.3	85.7	100.0	32.4	33.7	15.8	22.0	20.0	28.8
Y	13.7	15.8	12.3	14.0	17.5	14.9	19.1	26.9	25.0	30.4	26.2	26.6	100.0	63.1	15.3	16.8	21.2	19.3
LMP2	12.6	13.6	13.4	15.1	17.4	17.1	19.1	27.1	28.7	27.7	29.4	29.4	57.1	100.0	14.3	22.1	21.3	19.4
HC5	10.9	14.5	11.2	20.0	14.0	15.8	17.5	15.5	19.1	20.0	18.9	18.4	18.3	19.3	100.0	21.0	24.9	22.5
HC7-I	15.8	16.2	13.1	16.0	15.2	16.6	15.7	19.3	19.3	20.4	21.2	21.2	17.2	21.3	19.9	100.0	16.7	22.1
HC10-I	16.2	13.6	10.9	14.4	15.4	12.9	15.2	17.6	16.1	19.9	19.0	19.0	19.5	19.3	25.6	16.6	100.0	18.0
HN3	13.4	13.8	12.5	14.2	12.4	11.6	13.7	17.6	18.7	22.9	22.4	24.0	18.3	19.5	18.1	20.3	18.3	100.0

Figure 5. Similarities of primary structures of various human proteasome subunits. Percentages of overall amino acid sequence identity between proteasomal subunits were obtained by computer analysis (*black box*). The right side (*open box*) shows the percent identity confined to highly conserved amino acid regions—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), LMP2 (20-198), and Z (43-221)—in which numbers show amino acid residues (start end) in the calculation. For the sources of sequence data, see Akiyama et al. (28). The gaps inserted to achieve maximal sequence homology of amino acids were deleted in statistical analyses.

of LMP2 and LMP7 have been observed in various other types of cells (21, 28). The increases in the mRNAs for LMP2, LMP7, and MECL1 were observed after treatment with IFN-γ for about 12 h, suggesting acute responses to IFN-γ of these three proteasomal subunits (Fig. 6 A).

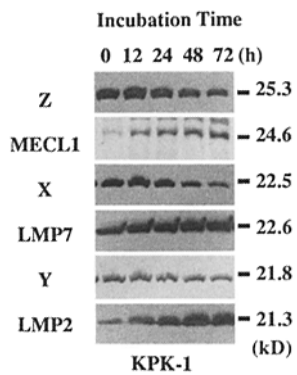Next, we examined whether other IFNs affected the expressions of these proteasomal genes. As shown in Fig. 6 B, IFN-α had no significant influence on the levels of expression of the genes examined, but IFN-β significantly enhanced the effect of IFN-γ on the expressions of LMP2 and LMP7 without affecting MECL1 expression in ACHN

and KPK-1 cells. This finding suggests that the mechanism regulating expression of subunit MECL1 may differ somewhat from those regulating subunits LMP2 and LMP7, although IFN-γ acts as a major inducer of all of them, and that the physiological role of proteasomes containing MECL1 may differ from that of proteasomes containing LMP2 and/ or LMP7. On the other hand, the amounts of the mRNAs of multiple α- and β-type subunits, including Z, X, and Y, were not affected appreciably by treatment with IFN-γ or other IFNs (Fig. 6).

We examined the effect of IFN-γ on the amounts of
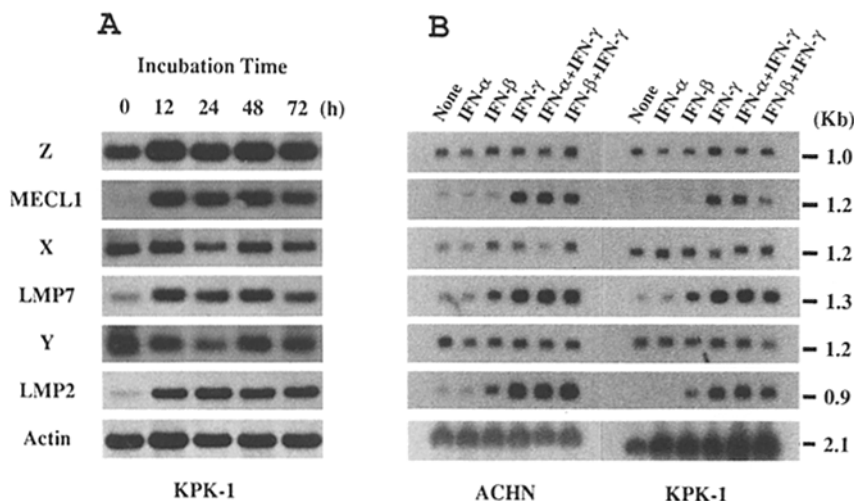
Figure 6. Effect of IFN-γ on expression of various proteasome subunit genes in various human cells. (*A*) KPK-1 cells were treated with IFN-γ (500 U/ml) for the indicated times, and then amounts of mRNAs encoding proteasomal proteins of Z, MECL1, X, LMP7, Y, and LMP2 were analyzed by RNA blot hybridization (21). (*B*) Effects of various IFNs on the expressions of subunits in renal carcinoma ACHN and KPK-1 cells. IFN-α (500 U/ml) and IFN-β (500 U/ml) were added individually or in combination with IFN-γ (500 U/ml) for 3 d.

Incubation Time

0 12 24 48 72 (h)

Z   – 25.3

MECL1   – 24.6

X   – 22.5

LMP7   – 22.6

Y   – 21.8

LMP2   – 21.3

KPK-1   (kD)

Figure 7. Immunoblot analyses of proteasomes with various antiproteasome antibodies in KPK-1 cells cultured with IFN-γ. Cells were treated with IFN-γ as in Fig. 6 A.

these proteasomal subunits. As shown in Fig. 7, immunoblot analysis showed marked increases of the LMP2, LMP7, and MECL1 proteins in KPK-1 cells, which roughly correlated with the amounts of their mRNAs (Fig. 6). However, neither the protein levels of X, Y, and Z nor the amounts of their mRNAs were greatly affected by IFN-γ (Fig. 1). This suggests that the complete loss or marked decrease in the expressions of subunits X, Y, and Z induced by IFN-γ is due to posttranslational degradation of X, Y, and Z, as proposed before (29, 30). However, the amounts of these three proteins were found to decrease gradually 2–3 d after stimulation by IFN-γ. Moreover, we showed previously that IFN-γ has no effect on the subunit pattern of preexisting proteasomes (21). Probably, although the induction of proteasomes containing LMP7, LMP2, and MECL1 by IFN-γ is rapid, the overall change from preexisting X/Y/Z-type proteasomes is slow, because of the large pool size and slow turnover of the latter, constitutive proteasomes (8, 51).

Discussion

Proteasomes containing LMP2 and LMP7 have been thought to be responsible for the MHC class I–restricted antigen-processing pathway, because transfection of LMP2 and LMP7 cDNAs altered the proteolytic specificity of proteasomes (52). Recently, we found that IFN-γ induced not only high syntheses of LMP2 and LMP7, but also marked reductions in the accumulation of two unidentified proteasome subunits, tentatively named X and Y (21, 27). This suggests the induction by IFN-γ of the replacements of proteasome subunits X and Y by their highly homologous subunits LMP7 and LMP2, respectively (28–30), which is presumably responsible for change of the functional diversity of proteasomes. In fact, cells from mice with a homologous deletion of the LMP7 show deficiencies in class I–restricted antigen presentation, which is due to reduced intracelluar antigen processing (23). Disruption of the LMP2 also resulted in defective cellular immunity under certain conditions, such as viral infections in gene knockout mice, although in intact animals, dysfunction of LMP2 did not have any appreciable effects (24). Apparently, LMP2 is necessary for the presentation of some antigens to cytotoxic T lymphocytes (53). On the other hand, the defect of antigen presentation in T2 cells, which have

deletions of not only the TAP1/2 genes but also the LMP2/LMP7 genes, can be suppressed almost completely by transfections of TAP1/2 genes into the mutant cells, suggesting that LMP2/LMP7 did not have much effect on the antigen-processing pathway (31–33). The latter findings suggest that there may be an additional proteasome gene(s) responsible for antigen processing mediated by the proteasome. Here we demonstrated the existence of a third pair of proteasome subunits reciprocally regulated by IFN-γ and propose that IFN-γ induces not only replacements of X and Y by LMP7 and LMP2, but also substitution of Z for MECL1, producing proteasomes responsible for antigen processing. Actually, IFN-γ alters the proteolytic specificity of proteasomes, increasing their activities for cleavage of peptide bonds on the carboxyl side of basic and hydrophobic amino acid residues of fluorogenic substrates, but decreasing their activities for peptides containing acidic amino acid residues (19–21). These changes of the proteasomal functions would be more appropriate for the generation of MHC class I–restricted peptides, because the carboxyl sides of antigenic peptides associated with the cell surface class I molecules consist mainly of hydrophobic and basic amino acid residues, not acidic amino acid residues (54). In contrast, reduction of chymotryptic activity by IFN-γ is reported by other investigators (25, 39), but the reason for these controversial results is unclear at present. It also remains unknown whether the new pair of substituted subunits, Z and MECL1, affects the proteolytic functions of proteasomes. This point is currently being examined.

The subunit organization of eukaryotic proteasomes is presumed to be $\alpha n(1\text{-}7)\beta n(1\text{-}7)\beta n(1\text{-}7)\alpha n(1\text{-}7)$, where n indicates the number of seven heterogeneous subunits (2). 7 human cDNAs encoding α-type subunits have been cloned, whereas 10 human cDNAs of β subunits, including Z, have been found so far (see Fig. 5). In this study, we suggest that IFN-γ may induce subunit replacements of X, Y, and Z by LMP7, LMP2, and MECL1, respectively, producing proteasomes that are perhaps more appropriate for the immunological processing of endogenous antigens, and so we propose the term immunoproteasomes for functionally distinct proteasomes induced by IFN-γ. Thus, in response to extracellular signals, such as IFN-γ, some β-type subunits appear to be replaced by very homologous, but different, gene products, which might be generated by gene duplication. Therefore, the total number of β-type subunits may have increased in multicellular organisms during evolution for acquisition of the MHC-restricted immune system. Previously, we proposed the name molecular adaptation for the interesting new phenomenon in which the proteasomal multisubunit complex acquires functional diversity by change in molecular composition in response to environmental stimuli (30). The subunit changes of proteasomes in various physiological conditions may be one reason why the proteasome has such a complex organization, but further analyses are required to determine their structural–functional relationships.

Proteasomes are present in both the nucleus and the cytoplasm of a variety of eukaryotic cells, and are localized

predominantly in the nuclei of various types of rapidly growing cells. We found that most of the α-type subunits contain nuclear location signal (NLS) and complementary NLS motifs (55). Thus different organizations of the α-type subunits may alter the intracellular localization of the proteasome. On the other hand, yeast genetical studies indicate that the β subunits are related with the active function, but the catalytic mechanism remains unsolved (49). Recently, it was demonstrated by x-ray crystallography (56) and site-directed mutagenesis (45) that the *T. acidophilum* proteasome is a novel threonine protease, the NH$_2$-terminal threonine residue of the β subunit, essential for the proteolytic function. Interestingly, three pairs of 10 homologous, but distinct, β-type subunits of eukaryotic proteasomes, that is, X/LMP7, Y/LMP2, and Z/MECL1 subunits but not the remaining four β subunits, have acquired a conserved NH$_2$-terminal threonine residue processed from their precursors, suggesting that they are active subunits. In considering these new facts, our previous finding for the cDNA cloning of X and Y (28) and the present observation regarding cDNA cloning of Z are very important, because they provide entirely new information suggesting that IFN-γ induces replacements of three pairs of homologous subunits with possibly catalytic functions, which may be responsible for the IFN-γ–dependent alteration of proteolytic specificity of the proteasome reported previously (19–21).

IFN-γ plays a central role in immunomodulatory functions through upregulation of the expressions of a variety of genes responsible for the immune response. As reported previously (21), IFN-γ had no effect on the cellular content of proteasomes. Therefore, it must modify the subunit organization and functions of proteasomes, resulting in accelerated or modulated processing of endogenous antigens.

This is a novel mechanism for the immunomodulatory action of IFN-γ, clearly differing from the previously known mechanism involving marked increases in the cellular concentrations of TAP and MHC molecules responsible for antigen presentation by cells, which is stimulated at the transcriptional level of these genes. Ma et al. (57) and Dubiel et al. (58) found an activator protein of the 20S proteasome, named PA28 (or 11S regulator). It is a homooligomeric complex that binds directly to both ends of the cylindrical 20S molecule (4, 59). Intriguingly, PA28 consists of two homologous proteins, named PA28α (equivalent to the originally described PA28/11S regulator) and PA28β (60), and the genes encoding these two activators have also recently been found to be responsive to IFN-γ (60, 61). In fact, PA28α is identical to a protein clones previously, named IGUP I-5111, which is one of the major gene products induced by IFN-γ in primary human keratinocytes (62). The purified PA28 protein greatly stimulates multiple peptidase activities of the 20S proteasome without affecting destruction of large protein substrates (57, 58), suggesting that it may cooperate with the 26S proteasome in a sequential proteolytic pathway. Thus, these newly identified IFN-γ–regulated activator genes in combination with three pairs of IFN-γ–regulated proteasome genes may provide answers to unsolved fundamental questions of redundancy in the antigen-processing pathway. As mentioned in the Introduction, the 26S proteasome is a large complex consisting of two functionally distinct units, a central cylindrical 20S proteasome with at least three distinct endopeptidase activities and regulatory parts attached to both ends of this central part that have ATPase and deubiquitinating actions (2, 4, 6, 9, 10). At present it remains unclear whether IFN-γ alters composition of the regulator complex.

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