Potential Immunocompetence of Proteolytic Fragments Produced by Proteasomes before Evolution of the Vertebrate Immune System

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Summary

To generate peptides for presentation by major histocompatibility complex (MHC) class I molecules to T lymphocytes, the immune system of vertebrates has recruited the proteasomes, phylogenetically ancient multicatalytic high molecular weight endoproteases. We have previously shown that many of the proteolytic fragments generated by vertebrate proteasomes have structural features in common with peptides eluted from MHC class I molecules, suggesting that many MHC class I ligands are direct products of proteasomal proteolysis. Here, we report that the processing of polypeptides by proteasomes is conserved in evolution, not only among vertebrate species, but including invertebrate eukaryotes such as insects and yeast. Unexpectedly, we found that several high copy ligands of MHC class I molecules, in particular, self-ligands, are major products in digests of source polypeptides by invertebrate proteasomes. Moreover, many major dual cleavage peptides produced by invertebrate proteasomes have the length and the NH_2 and COOH termini preferred by MHC class I. Thus, the ability of proteasomes to generate potentially immunocompetent peptides evolved well before the vertebrate immune system. We demonstrate with polypeptide substrates that interferon γ induction in vivo or addition of recombinant proteasome activator 28α in vitro alters proteasomal proteolysis in such a way that the generation of peptides with the structural features of MHC class I ligands is optimized. However, these changes are quantitative and do not confer qualitatively novel characteristics to proteasomal proteolysis. The data suggest that proteasomes may have influenced the evolution of MHC class I molecules.

T lymphocytes recognize peptide fragments of protein antigens presented on the cell surface by the class I and class II molecules of the MHC. The peptide fragments are generated proteolytically inside the cell. MHC class II molecules are loaded in a secretory compartment with peptides generated in endosomes. MHC class I molecules are loaded with peptides mainly generated in the cytoplasm and transported into the ER/*cis*-Golgi by the peptide transporter associated with antigen processing (TAP[1]; 1, 2). The vast majority of peptides presented by MHC molecules are derived from self-proteins. The peptide-binding grooves of class II molecules are open at both ends and bind peptides of heterogenous length (usually 12–25 amino acids [aa]); the peptide-binding grooves of class I molecules are closed at both ends and usually bind peptides of closely defined length (8–10, mostly 9 aa). In the latter case, the peptide is usually fixed by two allele-specific anchor residues that are complementary to allele-specific pockets in the MHC class I peptide-binding groove (3, 4). In addition, H bonds are formed between relatively invariant polar aa at the ends of the binding groove and the NH_2 and COOH termini of the peptide (5). Typically, one of the allele-specific pockets, the COOH-terminal F pocket, accommodates an aliphatic, aromatic, or positively charged aa at the COOH terminus of an octa/nonamer peptide. The second anchor may

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[1]*Abbreviations used in this paper:* β_2m, β_2 microglobulin; aa, amino acid; BTG1, B cell translocation gene 1; D^b, H-$2D^b$; hsp, heat shock protein; K^b, H-$2K^b$; LLnL, *N*-acetyl-l-leucinyl-l-Leucinal-l-norleucinal; MALDI-Tof-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PA, proteasome activator; SSU, small subunit; TAP, transporter associated with antigen processing.

reside at the second, the third, or the fifth position from the NH$_2$ terminus of the peptide, and is more variable (3).

For the proteolytic generation of MHC class I epitopes, the immune system of vertebrates appears to have recruited the proteasomes. These ubiquitous multi-subunit endoproteases are phylogenetically ancient, as they occur in archea and bacteria, as well in eukarya. In eukaryotic cells, proteasomes appear to be the major proteolytic system of the nucleus and the cytosol. Three main forms are observed. The 20S proteasome, by itself capable of degrading misfolded or damaged polypeptides, represents the proteolytic core of the larger and more complex 26S proteasomes and the 20S–proteasome activator (PA)28 complexes (6). It has a barrel-shaped hollow structure with four layers of rings, each composed of seven subunits. The outer rings consist of proteolytically inactive α-type subunits, the inner rings of β-type subunits. Archaebacterial proteasomes possess one type of proteolytically active β subunit, i.e., all seven members in a β ring have a NH$_2$-terminal threonine acting as the nucleophile in peptide-bond hydrolysis. In eukaryotes, three out of seven different β-type subunits contain such a site. Proteolysis takes places inside the central cavity, between the two β rings (7, 8). In vertebrates, the three proteolytically active β-type subunits (X, Y, and Z) have IFN-γ–inducible homologues (LMP7, LMP2, and MECL1) replacing their constitutive counterparts when induced. The PA28 proteasome activator, giving rise to the 20S–PA28 complexes, is IFN-γ–inducible as well. LMP2 and LMP7, but not MECL1 and PA28, are encoded by genes in the MHC (9).

The assembly of the class I heavy chain with β$_2$ microglobulin (β$_2$m) can be substantially inhibited by peptide aldehydes, potent but not absolutely specific proteasome inhibitors (10). This seminal information, together with the discovery of the MHC-encoded proteasome subunits (11), have stimulated a host of investigations into the role of vertebrate 20S proteasomes in the processing of antigens presented by MHC class I (for review see references 6 and 9). Although most of these studies converged in suggesting a major role for proteasomes in the generation of MHC class I epitopes, it is also clear that the evidence in support of this notion remains, to some extent, circumstantial. We have recently reported that the length distribution of dual cleavage proteolytic fragments produced by mouse 20S proteasomes centers around 8–11 mer. The frequencies of individual aa at the COOH termini of proteolytic fragments generated by proteasomes correlated strikingly with that at the corresponding positions of so far eluted MHC class I ligands. For the NH$_2$ termini too, a significant enrichment of small and polar aa was observed for both proteasomal degradation products and MHC class I ligands (12). Intriguingly, similar COOH and NH$_2$ termini as well as a similar length distribution were found for peptides preferentially transported by TAP or by certain TAP alleles (13, 14). Together, these results are consistent with the notion that many of the peptides transported by TAP and many of the epitopes presented by MHC class I are directly derived by proteasomal proteolysis.

The presently available data on the substrate/ligand specificities of proteasomes, TAP, and MHC class I, in addition to suggesting coordinated function of the three systems, argue for some degree of coevolution. It has been suggested that peptide binding to MHC class I and class II may have been determined by proteolytic pathways available in ancestors before emergence of the vertebrate immune system (15). Here, we examine the hypothesis that proteasome-mediated proteolysis may have influenced the evolution of MHC class I. This hypothesis requires that the capacity of proteasomes to generate fragments with the general structural features of MHC class I binding peptides is conserved and extends back in evolution to before the emergence of MHC and T cell recognition. Although structural homologies would anticipate a high degree of conservation in proteasomal functions, differences in cleavage site usage between proteasomes at different stages of evolution have been reported (16, 17). Furthermore, it has been suggested that the IFN-γ inducible elements drastically alter the repertoire of peptide products of proteasome mediated proteolysis (18, 19). Our results suggest that the capacity of proteasomes to generate potentially immunocompetent peptides, including the efficient generation of several proven MHC class I ligands, is highly conserved in eukaryotes and evolved before the vertebrate immune system. The functional modifications by the IFN-γ–inducible elements suggest an evolutionary adaptation of proteasomes to their novel immune functions. However, these modifications appear to be mainly quantitative in nature and did not confer fundamentally novel characteristics to proteasomal proteolysis.

Materials and Methods

Reagents, Cell Lines, and Antibodies. The protease inhibitor *N*-acetyl-l-leucinyl-l-leucinal-l-norleucinal (LLnL) was purchased from Sigma Chemical Co. (St. Louis, MO); IFN-γ was from Boehringer Mannheim GmbH (Mannheim, Germany). The proteasome inhibitor lactacystin was purified as described (20). The C57BL/6-derived thymoma EL4, the human lymphoblastoid cell line T1, the human erythroblastoid cell line K562, and *Drosophila melanogaster* Schneider cells were obtained from American Type Culture Collection (Rockville, MD). Monoclonal antibodies were prepared from the hybridoma Y3 (anti-H2 class I K^b heterodimers; 21). Rabbit antiserum specific for sequences encoded by exon 8 of the K^b gene and reactive with free or β$_2$m-associated K^b heavy chains was a gift from Dr. S. Nathenson (Albert Einstein College, New York).

Immunoprecipitation Experiments. EL4 cells (10^7 cells/ml) were incubated for 2 h at 37°C in the presence or absence of proteasome inhibitors in cysteine and methionine-free medium, and for the last 45 min of incubation, (^{35}S) cysteine/methionine (700 μCi/ml) was added. After metabolic labeling, cells were lysed in 0.5% Nonidet P-40 (ICN Biomedicals Inc., Plainview, New York) and 0.5% Mega 9 (Sigma Chemical Co.). Samples were precleared for 60 min at 4°C with protein A–Sepharose (Pharmacia, Uppsala, Sweden) pretreated with 1 mg/ml bovine serum albumin. For immunoprecipitation, either mAb Y3 (15 μg) or 4 μl rabbit α exon 8 antiserum were added to the precleared lysates for 2 h. For the last 90 min of incubation, protein A–Sepharose was added.

Immunoprecipitates were analyzed by SDS-PAGE on 12% gels. Quantitation of gel bands was performed with the aid of a Fujix BAS 1,000 phosphorimager.

Peptides and Protein Substrates. Peptides were synthesized by using solid-phase 9-fluorenylmethoxycarbonyl chemistry in a peptide synthesizer (431A; Applied Biosystems, Foster City, CA) and subsequently purified by reverse phase HPLC. The identity of peptides was established by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-Tof-MS) and aa sequence analysis on Hewlett-Packard Co. (Palo Alto, CA) instruments. Ribulose 1,5 bisphosphate carboxylase small subunit was purified from intact pea chloroplasts by denaturing continuous electrophoresis (Grimm, R., manuscript submitted).

Purification of 20S Proteasomes and of Recombinant PA28α. 20S proteasomes were purified from EL4 cells cultured with or without 50 U/ml IFN-γ for 6 d, from K562, T1, and *D. melanogaster* Schneider cells, as well as from *Saccharomyces cerevisiae* (strain YRG-2) by fractionated precipitation of the cytosol with polyethylene glycol 6,000 followed by anion exchange chromatography on a Mono Q column (HR 5/5; Pharmacia) as previously described for EL4 cells (22). Modifications of the NaCl gradient (buffer A: 20 mM Tris/HCl [pH 7.2]; buffer B: 20 mM Tris/HCl [pH 7.2], 1 M NaCl) were as follows: *S. cerevisiae*: 0–38% B in 50 min, 38–48% B in 25 min, proteasomes eluted at 45% B; *D. melanogaster*: 0–28% B in 50 min, 28–31% B in 25 min, proteasomes eluted at 30% B; IFN-γ–treated EL4 cells: 0–34% B in 65 min, 34–37% B in 40 min, proteasomes eluted at 36% B; K562 cells: 0–37% B in 55 min, 37–41% B in 40 min, proteasomes eluted at 38% B; T1 cells: 0–35% B in 55 min, 35–38% B in 40 min, proteasomes eluted at 37% B. T1 proteasomes were further purified on a Phenylsuperose column (Pharmacia). The proteasomes were recovered in the flow-through fraction. 20S proteasomes from *Rhodococcus* sp. and recombinant *Thermoplasma acidophilum* proteasomes were purified as described (23, 24). The purity of proteasomes was assessed by SDS-PAGE followed by silver staining as described (22). Purification of recombinant human red blood cell PA28α is described in references 25 and 26.

Proteasome Digests and Analyses. Digestions of synthetic peptides (6 μg) and of the small subunit of ribulose 1,5 bisphosphate carboxylase (10 μg) with isolated proteasomes (1 μg) were performed at 37°C, except in the case of *Thermoplasma* proteasomes at 60°C, in a total volume of 300 μl buffer (20 mM Hepes/KOH [pH 7.0] 1 mM EGTA, 0.5 mM EDTA, 5 mM MgCl$_2$, 0.5 mM 2-mercaptoethanol, 0.02% sodium azide). Digestions of ovalbumin fragments Ova$_{37-77}$ and Ova$_{239-281}$, and of the protein substrate were done in the presence of 0.004% SDS. In the experiments shown in Fig. 7, recombinant PA28α was added in a fivefold molar excess over the proteasome. Aliquots of the reaction mixture were separated by reverse phase HPLC on a SMART system equipped with a μRPC C2/C18 SC 2.1/10 column (Pharmacia). Eluent A was 0.1% (vol/vol) TFA/water; eluent B was 80% (vol/vol) acetonitril/water (0.081% TFA). The identity of peptides in individual HPLC fractions was established by MALDI-Tof-MS and aa sequence analysis by Edman degradation.

For molecular mass determination without separation of the peptide products, the reaction mixtures were desalted with the help of a mini-C18 column. The peptides were then eluted by the addition of 60% acetonitril in water, concentrated, and then directly analyzed with a 62025A Maldi-Tof-MS (Hewlett-Packard Co.) using dihydroxybenzoic acid as the matrix.

For proteasome cleavage site determination, aliquots of the reaction mixtures were directly subjected to aa sequence analysis by Edman degradation (pool sequencing). The sites and efficiencies of cleavage were determined from the sequence cycles and relative yields of amino acids unique in the analyzed sequence. For example, if the unique amino acid *X* appears in cycle *Y* of Edman degradation, a cleavage site is revealed *Y* residues from amino acid *X* towards the NH$_2$ terminus; if the unique amino acid appears in more than one cycle, several cleavage sites are revealed, their relative strengths corresponding to the relative yields of the aa in each cycle.

Results

Marked Inhibition of the Assembly of MHC Class I Molecules by the Proteasome Inhibitor Lactacystin. A functional pressure of proteasome-mediated proteolysis on the evolution of MHC class I could be envisaged if proteasomal digestion provided the major source of peptides for assembly with MHC class I. We examined the assembly of MHC class I–peptide complexes in the presence of the proteasome inhibitor lactacystin (27). Except proteasome-related particles in bacteria (28), no other protease has been reported to be inhibited by this compound. The murine thymoma cell line EL4, expressing the class I molecules H-2K^b (K^b) and H-2D^b (D^b), was incubated with or without lactacystin and metabolically labeled with ^{35}S-methionine plus cysteine. Immunoprecipitation was performed either with mAb Y3, detecting a conformational determinant of K^b present only on the assembled trimolecular complex, or with an antiserum (anti–exon 8 antiserum) that recognizes the cytoplasmic tail of K^b and D^b molecules, independent of whether a peptide is bound or not. As shown in Fig. 1, addition of lactacystin caused a marked decrease in the amount of K^b molecules precipitable with the conformation-specific antibody, but not of that precipitated by the anti–exon 8 reagent. Plateau inhibition was at ~67% as calculated by phosphorimaging (see Materials and Methods), similar or slightly better than
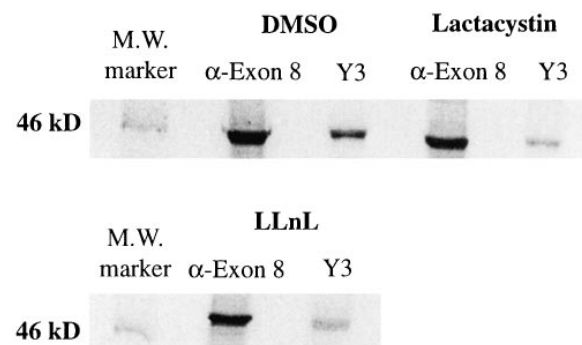
Figure 1. Lactacystin inhibits the assembly of K^b class I molecules. Autoradiograms of immunoprecipitated K^b heavy chains from ^{35}S-labeled EL4 cells. EL4 cells (10^7) were mock-treated for 2 h with DMSO, or with LLnL (100 μM), or with various concentrations of lactacystin. Concentrations of lactacystin between 100 and 1,000 μM gave maximal inhibition. Only one concentration (250 μM) is shown. [^{35}S]methionine/cysteine (0.7 mCi/ml) was added for the last 45 min of incubation. Detergent lysates were precleared and immunoprecipitated with either the conformation-dependent anti-K^b mAb Y3 or a conformation-independent anti-K^b exon 8 immunoglobulin preparation. Immunoprecipitates were separated by a 12% SDS-PAGE gel and autoradiographed.
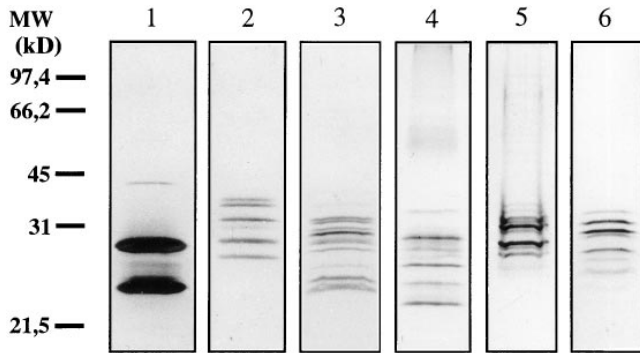
Figure 2. Polyacrylamide gel electrophoresis of purified proteasomes. Proteasomes were isolated from various organisms or cell lines as described in Materials and Methods and analyzed by SDS-PAGE on 12% gels followed by silver staining. The low molecular mass polypeptides between 22 and 35 kD represent the subunits of 20S proteasomes. Lane *1*, *Thermoplasma acidophilum*; lane *2*, *Rhodococcus sp.*; lane *3*, *S. cerevisiae*; lane *4*, *D. melanogaster*; lane *5*, mouse EL4 cells; lane *6*, human T1 cells.

that reported for the peptide aldehyde inhibitor LLnL (Fig. 1; reference 10). These data are in agreement with recent results on the inhibition of antigen presentation by lactacystin (29) and further support the notion that proteasomes participate in the generation of the majority of peptides presented by MHC class I.

Efficient Generation of Proven MHC Class I Ligands by Proteasomes of Eukaryotic Invertebrates. At early time points in the processing of short polypeptide substrates by isolated mouse 20S proteasomes, single cleavage intermediates can be detected in addition to dual cleavage oligopeptides. After consumption of the original substrate and of the single cleavage intermediates, the reaction appears to slow down and the mixture of peptide fragments approaches a relatively stable state (12). We believe that the stable state in vitro most closely resembles the conditions in vivo. Therefore, although several time points have been analyzed to compare 20S proteasomes at distinct stages of evolution (Fig. 2), we present data on the relatively stable product patterns determined after complete substrate turnover.

We have previously shown that the immunodominant ovalbumin epitope $Ova_{257-264}$ (SIINFEKL; reference 30) is the major stable product generated by mouse 20S proteasomes from the 22-mer $Ova_{Y249-269}$ as well as from the 44-
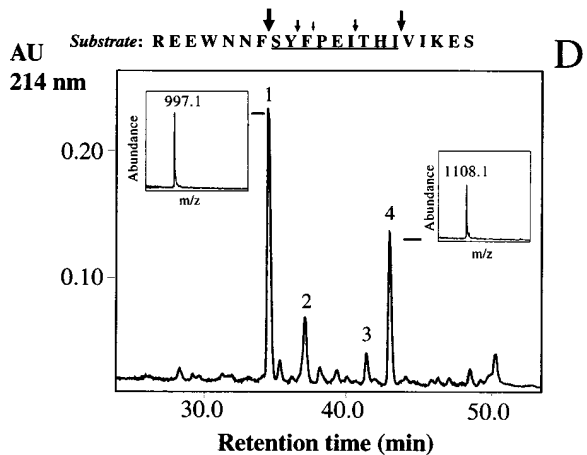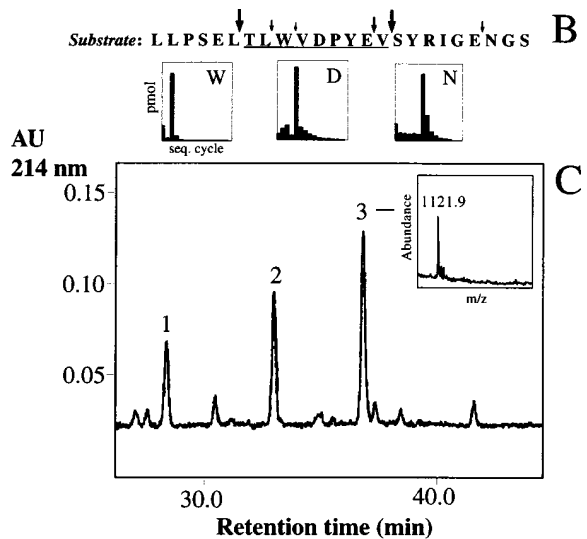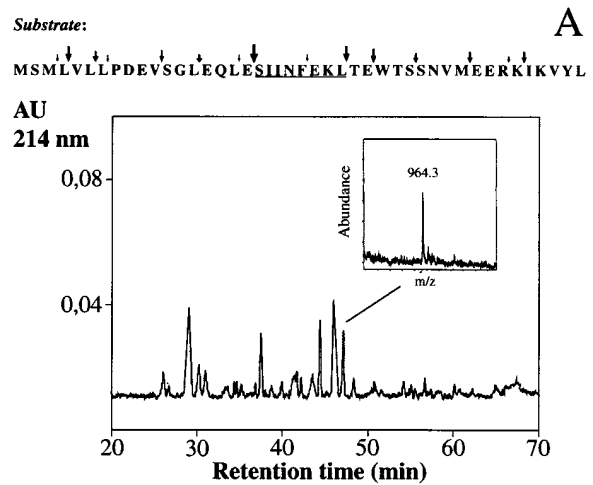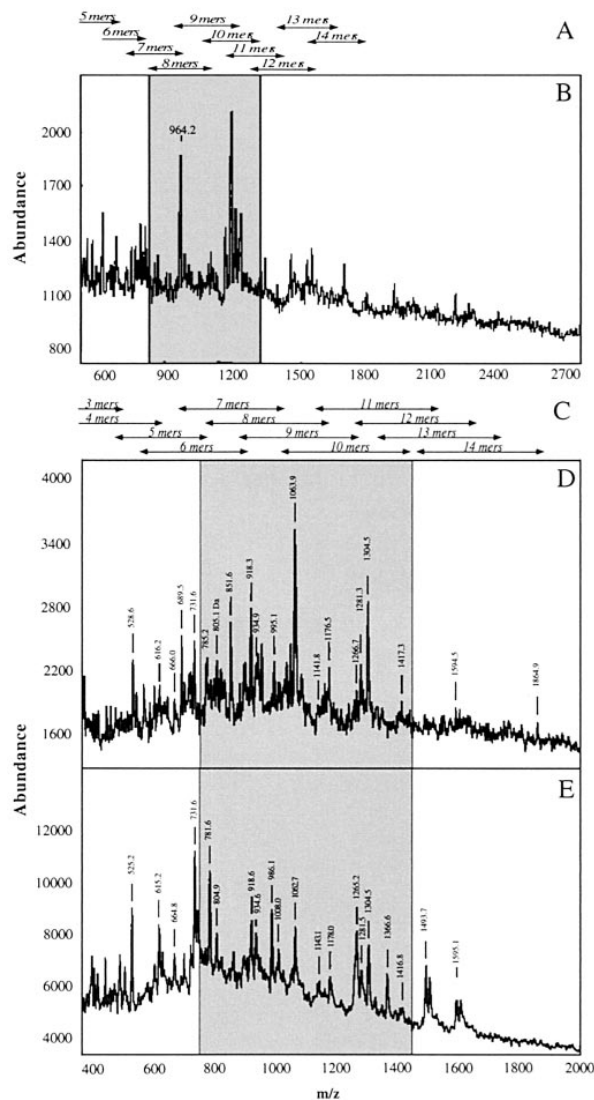
Figure 3. Proteasomes from eukaryotic invertebrates have the capacity to efficiently generate known MHC class I ligands. (*A*) Digestion of the 44-mer $Ova_{239-281}$ with 20S proteasomes isolated from *D. melanogaster* Schneider cells. The proteasome cleavage sites indicated by the arrows above the sequence were determined by Edman degradation pool sequencing (for raw data see Fig. 5 *B*). Reverse phase HPLC chromatogram of the peptide mixture is shown below. The mass spectrometry inset refers to the peak that contains the immunodominant CTL epitope SIINFEKL. (*B* and *C*) Digestion of the 24-mer $BTG1_{97-120}$ with 20S proteasomes isolated from *S. cerevisiae*. (*B*) Proteasome cleavage site determination by Edman degradation pool sequencing. For interpretation of pool-sequencing data, see legend to Fig. 5. (*C*) Reverse phase HPLC chromatogram. The numbered peaks contain the peptides LLPSEL (*1*), TLWVDPYE (*2*), and the HLA A2.1 ligand TLWVDPYEV (*3*). (*D*) Digestion of the 21-mer

$JAK1_{348-368}$ with 20S proteasomes isolated from *D. melanogaster* Schneider cells. Proteasome cleavage sites indicated by the arrows above the sequence were determined from the peptide products identified in the reverse phase HPLC chromatogram shown below. The numbered peaks contain the peptides REEWNNF (*1*), REEWNNFSY (*2*), SYFPEI (*3*), and the K^d ligand SYFPEITHI (*4*). All peptide mixtures shown were analyzed after substrate consumption. The peptides contained in the peaks marked with numbers and/or mass spectrometry inserts were identified by Maldi-Tof-MS (insets) and Edman degradation (not shown).

mer Ova$_{239-281}$ (12). Here we show that this octamer is also a dominant product of digestion of Ova$_{239-281}$ by 20S proteasomes isolated from *D. melanogaster* Schneider cells (Fig. 3 *A* and Fig. 4 *A*). As a second example, we studied the generation of the nonamer TLWVDPYEV, an endogenous peptide derived from the product of the antiproliferative B cell translocation gene 1 (BTG1) and eluted as a major self-epitope from the human class I molecule HLA-A2.1 (31). Fig. 3, *B* and *C* show that this nonamer peptide is the major dual cleavage product generated by yeast (*S. cerevisiae*) proteasomes of the synthetic 24 mer encompassing this peptide in the sequence of BTG1. Moreover, we studied a 21-mer sequence derived from the tyrosine kinase JAK1 containing the nonamer SYFPEITHI, the most abundant self-peptide presented by mouse H-2K^d molecules of P815 cells (32, 33), and previously shown to be generated by digestion with mouse 20S proteasomes (34). We detected the epitope as the predominant dual cleavage product of the 21 mer with *Drosophila* proteasomes (Fig. 3 *D*). Thus, proteasomes from invertebrate eukaryotes have a high potency to generate proteolytic fragments that have been proven to serve as ligands of MHC molecules in the vertebrate immune system.
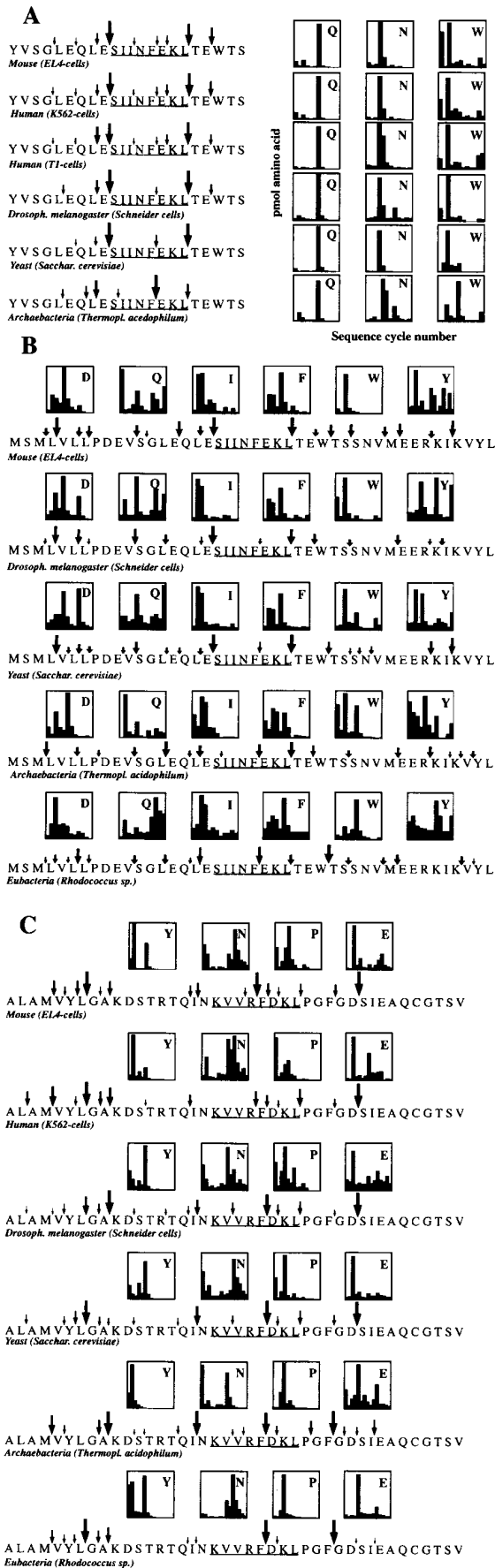
The Majority of Dual Cleavage Peptides Produced by Invertebrate Proteasomes Are in Size Range of MHC Class I Ligands. More than 90% of the peptides so far eluted from MHC class I molecules are 8–10 aa in length (3). We have recently shown that more than half of the dual cleavage proteolytic fragments generated by digestion of Ova$_{239-281}$ with mouse 20S proteasomes are 8–11 aa in length (12). In Fig. 4, *A* and *B*, we show that the peptides most efficiently produced from this substrate by *Drosophila* 20S proteasomes are in the size range of MHC class I–binding peptides (Fig. 4 *B*, *shaded area*). In addition, we studied a longer and immunologically undefined substrate, the 123-aa small subunit (SSU) of ribulose 1,5 bisphosphate carboxylase from the garden pea (*Pisum sativum L.*) (Fig. 4, *C–E*). Digests were prepared with proteasomes from mouse EL4 cells and from yeast. Most of the abundant masses in the digests represented peptide sizes of 5 to 11 aa for EL4 proteasomes or 5 to 13 aa for yeast proteasomes. In both cases, the majority of the peptides were 8–10 mer (Fig. 4, *D* and *E*, *shaded areas*). The masses of a number of abundant products are identical or nearly identical in the digests in Fig. 4, *D* and *E*, indicating that many of the peptides produced from SSU by mouse and yeast proteasomes may be identical.

Conserved General Cleavage Site Specificity of Proteasomes from Vertebrates and from Eukaryotic Invertebrates. We studied the cleavage site (...P$_3$P$_2$P$_1$ − P$_1'$P$_2'$P$_3'$...) preferences in polypeptides of 20S proteasomes isolated from a variety of organisms including archaebacteria, eubacteria, and nonvertebrate and vertebrate eukaryotes (see Fig. 2). Fig. 5 *A* shows the results obtained by pool sequencing of digests of the 22-mer Ova$_{Y249-269}$ containing the immunodominant Ova$_{257-264}$ (SIINFEKL) epitope. The cleavage patterns of all proteasomes of eukaryotic origin, including the murine cell line EL4, the human cell lines T1 and K562, as well as of insects and of yeast are remarkably similar; the predominant

Figure 4. Most peptides efficiently produced by 20S proteasomes from invertebrate eukaryotes fall into the size range of MHC class I ligands. The 44-mer peptide Ova$_{239-281}$ (*A* and *B*) and the 123-aa SSU of ribulose 1,5 bisphosphate carboxylase (*C*, *D*, and *E*) were digested with isolated proteasomes. After substrate consumption, the resulting peptide mixtures were subjected to MALDI-Tof-MS. (*B*) Mass spectrum of the peptide mixture obtained upon digestion of Ova$_{239-281}$ with *D. melanogaster* proteasomes. The mass peak representing the immunodominant OVA epitope SIINFEKL (theoretical mass: 964.3) is indicated. (*D* and *E*) Mass spectra of the peptides mixtures obtained upon digestion of SSU with proteasomes isolated from the murine cell line EL4 (*D*) and from yeast (*E*). The shaded areas in *B*, *D* and *E* mark the mass range of 8–10-mer peptides, i.e., the typical size range of MHC class I ligands. The mass ranges of all overlapping peptides of Ova$_{239-281}$ and SSU are indicated in *A* and *C*, respectively. In the SSU digests with proteasomes isolated from EL4 cells (*D*) or from yeast (*E*), 56.7 and 58.6%, respectively, of all discernible masses fell into the size range of 8–10-mer peptides.

cleavage sites reside after the same hydrophobic (L$_{264}$-T$_{265}$) and acidic (E$_{256}$-S$_{257}$) aa. These cleavage sites precisely coincide with the NH$_2$- and COOH-terminal epitope boundaries. In contrast, the cleavage pattern of archaebacterial proteasomes is clearly different; they prefer to cleave after

A

YVSGLEQLESIINFEKLTEWTS
Mouse (EL4-cells)

YVSGLEQLESIINFEKLTEWTS
Human (K562-cells)

YVSGLEQLESIINFEKLTEWTS
Human (T1-cells)

YVSGLEQLESIINFEKLTEWTS
Drosoph. melanogaster (Schneider cells)

YVSGLEQLESIINFEKLTEWTS
Yeast (Sacchar. cerevisiae)

YVSGLEQLESIINFEKLTEWTS
Archaebacteria (Thermopl. acedophilum)

pmol amino acid

Q N W

Sequence cycle number

B

D Q I F W Y

MSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYL
Mouse (EL4-cells)

MSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYL
Drosoph. melanogaster (Schneider cells)

MSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYL
Yeast (Sacchar. cerevisiae)

MSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYL
Archaebacteria (Thermopl. acidophilum)

MSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYL
Eubacteria (Rhodococcus sp.)

C

Y N P E

ALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSV
Mouse (EL4-cells)

ALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSV
Human (K562-cells)

ALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSV
Drosoph. melanogaster (Schneider cells)

ALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSV
Yeast (Sacchar. cerevisiae)

ALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSV
Archaebacteria (Thermopl. acidophilum)

ALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSV
Eubacteria (Rhodococcus sp.)

aromatic (F_{261}-E_{262}) and aliphatic aa (L_{264}-T_{265}, L_{255}-E_{256}), no cleavage after acidic aa is seen, and the major cleavage site destroys the epitope. Analyses of the degradation of longer substrates is shown in Fig. 5, *B* and *C*. The 44-mer Ova$_{239-281}$ (Fig. 5 *B*) represents a longer fragment containing the immunodominant SIINFEKL; the 41-mer Ova$_{37-77}$ (Fig. 5 *C*) contains the poorly immunogenic epitope Ova$_{55-62}$ (KVVRFDKL), also presented by K^b (22, 35). For bacterial proteasomes, the data in Fig. 5 *C* highlight the preference for aromatic and aliphatic aa in P_1, whereas cleavage after charged aa is rare but not impossible (Fig. 5 *B*). Cleavage patterns of all eukaryotic examples, although not fully identical, reflect the same broad but characteristic P_1 specificity spectrum. About 60–65% of the peptide bonds hydrolyzed (e.g., 11/17 in Ova$_{239-281}$ by mouse EL4-proteasomes; 11/18 by yeast proteasomes) have an aromatic or a hydrophobic aliphatic aa in the P_1 position. Most of the remaining peptide bonds have either a positively (R) or negatively charged (E, D) aa in the P_1 position. In addition, together with results in Fig. 3 (see above), these data extend to invertebrate eukaryotes our previous finding that proteasomes have a preference for small or polar aa in the $P_{1'}$ position of the scissile bond (12). Major cleavage sites are: E_{256}-S_{257} and L_{264}-T_{265} in Ova$_{Y249-269}$ (Fig. 5 *A*), L_{102}-T_{103} and V_{111}-S_{112} in BTG1$_{97-120}$ (see Fig. 3, *B* and *C*), and F_{354}-S_{355} in JAK1$_{348-368}$ (see Fig. 3 *D*).

Functional Effects of IFN-γ–Inducible Elements of Vertebrate Proteasomes. The existence of IFN-γ–inducible proteasomal elements in vertebrates indicates that the proteasomes themselves have evolved by adapting to their novel immunological role. If the specificity of proteasomal proteolysis was drastically and qualitatively altered by the IFN-γ–inducible elements, a significant restricting role of proteasomes in the evolution of MHC would be less likely. Most previous studies on the functional effects of the IFN-γ–inducible β subunits LMP2, LMP7, and MECL1 used short (3–4 aa) fluorogenic substrates, and inconsistent changes in peptidase activities have been reported by different investigators. By digesting polypeptide sequences more likely to resemble physiological proteasome substrates, we compared proteasomes from untreated with that of IFN-γ–treated EL4

Figure 5. Proteasomes from vertebrates and from eukaryotic invertebrates show highly conserved cleavage patterns in polypeptides. Synthetic peptides Ova$_{Y249-269}$ (*A*), Ova$_{239-281}$ (*B*), and Ova$_{37-77}$ (*C*) were incubated in the presence of 20S proteasomes isolated from the indicated cell lines or organisms. After substrate consumption, the mixtures were subjected to pool sequencing by Edman degradation. Proteasome cleavage sites were determined and quantitatively estimated by the sequence cycle numbers and the yields of unique amino acids. For example, the strong signal for asparagine (*N*) in sequence cycle *4* (panel *A*) indicates a strong cleavage site four residues towards the NH$_2$ terminus of N between E-S. Although isoleucine (*I*) is not unique, these signals are necessary for the interpretation of the phenylalanine (*F*) signal, and are therefore included in panel *B*. I undergoes racemization to iso- and alloleucine, the latter representing 30–40% and coeluting with F. The phenylalanine signals in cycles 2 and 3 are therefore caused by isoleucine (e.g., EL4 digest). Cleavage sites are indicated by arrows, with the sizes reflecting estimates of the relative efficiency of cleavage. The CTL epitopes SIINFEKL and KVVRFDKL are underlined.
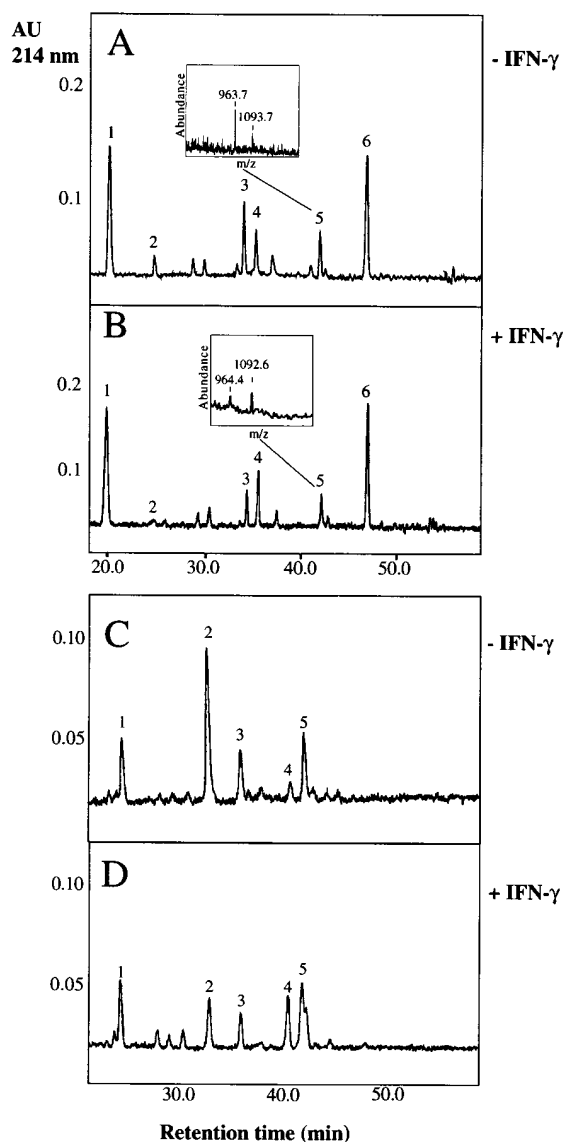
Figure 6. Proteasomes from IFN-γ–treated cells generate more peptides with hydrophobic and fewer peptides with acidic COOH termini from polypeptides. The substrates $Ova_{Y249-269}$ (*A* and *B*) and $BTG1_{97-120}$ (*C* and *D*) were incubated in the presence of 20S proteasomes from untreated EL4 cells (*A* and *C*) or EL4 cells treated with IFN-γ (*B* and *D*). The sequences of the substrates are given in Fig. 5, *A* and *B*, respectively. After consumption of the substrates, the peptide mixtures were separated by reverse phase HPLC. Peptides contained in the peaks marked with numbers are: (*A* and *B*) TEWTS (*1*), YVSGLE (*2*), YVSGLEQLE (*3*), YVSGLEQL (*4*), ESIINFEKL and the K^b ligand SIINFEKL (*5*), and SIINFEKLTEWTS/ESIINFEKLTEWTS (*6*); (*C* and *D*) LLPSEL (*1*), TLWVDPYE (*2*), TLWVDPYEVS (*3*), the HLA-A2.1 ligand TLWVDPYEV (*4*), and TLWVDPYEVSY (*5*). Peptides were identified by MALDI-Tof-MS and Edman degradation.

cells. Enhanced expression of LMP2 and LMP7 in induced compared to uninduced cells was monitored by Western blot analyses (not shown). Fig. 6, *A* and *B* show the HPLC patterns obtained upon digestion of $Ova_{Y249-269}$, Fig. 6, *C* and *D* that of the BTG1-derived 24 mer. In both cases, we observe that proteasomes from IFN-γ–treated cells generate

increased amounts of fragments with hydrophobic COOH termini (YVSGLEQL is peak *4* in Fig. 6, *A* and *B*, and TLWVDPYEV is peak *4* in Fig. 6, *C* and *D*) and decreased amounts of fragments with acidic COOH termini (YVSGLE is peak *2*, and YVSGLEQLE is peak *3* in Fig. 6, *A* and *B*; TLWVDPYE is peak *2* in Fig. 6, *C* and *D*). This is in line with general preferences of MHC class I molecules, although it does not necessarily result in improved production of each individual epitope; production of $BTG1_{103-111}$ (TLWVYPDEV, peak *4* in Fig. 6, *C* and *D*) is improved, whereas production of $Ova_{257-264}$ (SIINFEKL, contained in peak *5*, theoretical mass: 963.14, in Fig. 6, *A* and *B*) is impaired. In spite of these quantitative changes, however, the same set of major proteolytic fragments is produced by proteasomes isolated from uninduced and from IFN-γ–induced cells.

The IFN-γ–inducible PA28 enhancer, existing in two homologous forms, α and β (25, 36), binds as a ring-like hexa/heptameric structure to the α endplates of the 20S proteasome (37). PA28-capped 20S proteasomes exhibit enhanced activity towards short fluorogenic model substrates (26, 38–40). Here we analyze the effects of recombinant PA28α on the digestion of the 22-mer $Ova_{Y249-269}$ by 20S proteasomes (Fig. 7). Addition of PA28α leads to a slightly increased turnover of the substrate and reverses the ratio between HPLC peak *4* (SIINFEKL and ESIINFEKL) and HPLC peak *5* (SIINFEKLTEWTS and ESIINFEKLTEWTS). The peptides in peak *4* represent dual cleavage fragments; those in peak *5* are produced by a single cleavage only. Thus, in the presence of PA28α, the rate of accumulation of dual cleavage peptides is relatively increased, in line with results recently reported for native PA28 presumably consisting of both α and β isoforms (34). However, the dual cleavage peptides that are more efficiently produced in the presence of the activator are generated also in its absence. Together, these results support the notion that the IFN-γ–inducible proteasomal elements, including PA28 as well as the inducible β-type subunits, modify the 20S-proteasome in such a way that the generation of immunocompetent peptides is quantitatively improved, without drastic alterations of the specificity of proteasomal proteolysis.

Discussion

The work presented in this paper was stimulated by our observation that each of three proven MHC class I ligands was found as a major proteolytic fragment upon digestion of precursor polypeptides with yeast and/or insect proteasomes. One possible way to rationalize this observation was that the preexisting proteolytic fragments of proteasomes provided an important evolutionary force in shaping the peptide binding groove of MHC class I molecules. Such a mechanism would predict that proteasomal digestion would be the major source of MHC class I ligands in immunologically competent vertebrates. The evidence in favor of this notion is in part circumstantial and is a matter of continu-
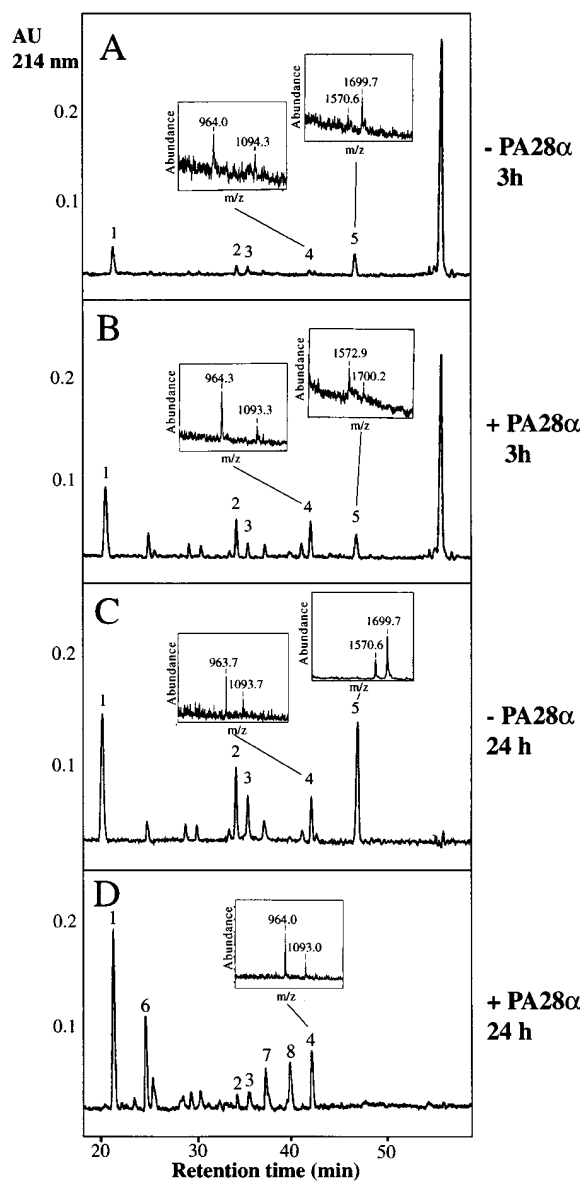
Figure 7. PA28α enhances the rate of accumulation of dual cleavage products without changing the cleavage site specificity of the proteasome. The substrate Ova$_{Y249-269}$ (for the sequence, see Fig. 5 *A*) was incubated with 20S proteasomes from EL4 cells in the absence (*A* and *C*) or presence (*B* and *D*) of recombinant PA28α. At the time points indicated, the mixtures were separated by reverse phase HPLC. The peptides in the peaks marked with numbers are: TEWTS (*1*), YVSGLEQLE (*2*), YVS-GLEQL (*3*), ESIINFEKL and the K^b ligand SIINFEKL (*4*), SIINFEK-LTEWTS and ESIINFEKLTEWTS (*5*), YVSGLE (*6*), SIINFE (*7*), and SIINF (*8*). The large peak at the right of *A* and *B* is undigested substrate. Peptides were identified by MALDI-Tof-MS and Edman degradation.

ing debate. We show that lactacystin, specific proteasome inhibitor until otherwise demonstrated, inhibits the assembly of about two-thirds of newly synthesized MHC class I heavy chains with β$_2$m. Inhibition of recognition of a panel of cytotoxic T lymphocyte epitopes by lactacystin has recently been reported (29). Although protease inhibitors cannot provide definitive proof, the combined data on this and other (10) proteasome inhibitors suggest that protea-

somes are critically involved in the generation of peptides for assembly with MHC class I. In addition, data from this and other laboratories (12, 34, 41) suggest that proteasomes often are involved in the final proteolytic steps of epitope generation.

A given MHC class I molecule can accommodate large arrays of different peptides. Typically, peptide specificity is constrained by two binding pockets, whereas the rest of the peptide sequence may vary. This is reminiscent of many endoproteases whose active sites can accommodate many different peptide sequences. In many of these enzymes, the primary determinant of substrate specificity is the S$_1$ subsite, accommodating the P$_1$ residue of the scissile bond P$_1$–P$_1'$. The F pocket of MHC class I molecules accommodates the side chain of the aa at the COOH terminus of the peptide with a strong preference for aliphatic, aromatic, and charged aa. Except for negatively charged residues, the P$_1$ preference of proteasomes closely resembles the preferences of the F pocket. The F pocket may therefore represent the structural center of the relationship of MHC class I molecules to the proteasome. Nevertheless, no class I allele has been found so far whose F pocket prefers peptides with acidic COOH termini, although these are also efficiently produced by proteasomes of vertebrates and invertebrates. This may be taken as an indication that MHC class I molecules evolved independently of proteasomes and that their preference for aliphatic, aromatic, or positively charged COOH termini has other reasons. However, it is also possible that there was selection against acidic amino acid side chains in the COOH-terminal ligand position. For example, it is conceivable that an acidic COOH-terminal aa side chain interferes with the formation of the H-bond system between conserved polar residues of class I and the free backbone carboxylate of the ligand. During the approach of a peptide with an acidic COOH-terminal aa side chain, the side-chain carboxylate, instead of the backbone carboxylate, may become engaged in H-bond formation, preventing proper anchoring of the ligand in the pockets of the peptide binding groove. Furthermore, peptides with acidic COOH termini may not be correctly handled by other components of the processing and presentation machinery, such as heat shock proteins (hsp), which have been proposed to shuttle peptides in the class I pathway (42), or TAP. However, at least human TAP has been shown to translocate peptides with acidic COOH termini (43).

Our pool-sequencing data strongly support the high degree of conservation among eukaryotes of the cleavage site usage in polypeptides by 20S proteasomes. Although cleavage efficiencies of individual peptide bonds are not always identical, the P$_1$ specificities of both vertebrate and invertebrate eukaryotic proteasomes are confined by the same broad, but well-defined, limits. The same applies to the P$_1'$ position where we frequently see small or polar side chains, particularly in major cleavage sites. For example, in the BTG1-derived sequence, yeast 20S proteasomes most efficiently hydrolyzed bonds that contain a small and/or polar P$_1'$ residue (L-T, V-S), in addition to a suitable P$_1$ residue. Similar preferences were seen in Ova$_{Y249-269}$ (E-S, L-T) and

JAK1$_{348-368}$ (F-S). Statistical analysis of so far eluted MHC class I ligands revealed an enrichment of small and polar residues in the NH$_2$-terminal position (most significantly S, but also G and A) (12). In addition, a recent analysis of D^b-binding ligands has shown a significant enrichment for S, T, and C in the NH$_2$-terminal position of high affinity peptides (44). Moreover, small polar peptide NH$_2$ termini favor hydrogen bond formation in HLA-B27 (5). Also, TAP is known to have enhanced binding/translocation efficiency for peptides with small or polar residues (45; P. van Endert, personal communication). We favor the hypothesis that these structural features of the peptide-binding groove of MHC class I, and perhaps also of the peptide-binding site of TAP, may represent evolutionary adaptations to conserved features of proteasomal proteolysis.

A striking property of proteasomes is the defined length of their proteolytic fragments. For archaebacterial proteasomes, peptide length centers around 7 and 8 mers (46). Upon digestion of 22- and 44-mer OVA-derived polypeptides by mouse 20S proteasomes, we have recently observed that the majority of the dual cleavage peptide products were 8–11 mer. Peptides of this length dominated among the dual cleavage products at all time points tested, including early in the time course. Many of the peptides of this length are relatively stable, even upon prolonged digestion by proteasomes (12). Most MHC class I molecules bind 8–10/11-mer peptides (3). Nonamers seem to be preferred by most alleles. In this report, we show that a major proportion of the stable peptides generated by yeast and *D. melanogaster* proteasomes fall into the size range preferred by MHC class I. Thus, the putative evolutionary relationships between proteasomes and MHC class I may include the length of the peptide-binding groove. In addition, the highly conserved clusters of polar aa at both ends of the peptide binding groove may have evolved to facilitate efficient H-bond formation with the ends of the short peptides produced by proteasomes.

IFN-γ is a pivotal cytokine in the function of the immune system and the incorporation of IFN-γ–inducible elements into the structure of proteasomes is highly suggestive of an evolutionary adaptation to the requirements of the immune system. One of the IFN-γ–inducible β subunits, LMP7, is first detected in the nurseshark (47), i.e., at the same phylogenetic step as most other elements of the vertebrate immune system (48). It was therefore possible that MHC molecules evolved independently of proteasomes followed by a unidirectional adaptation of proteasomes to the requirements of MHC class I. On the other hand, as shown in the present paper, the ability of proteasomes to generate potentially immunocompetent peptides preceded the evolution of the MHC, suggesting the reverse order of adaptation. We were therefore interested in understanding the extent of functional modification inflicted upon proteasomes by IFN-γ–inducible elements.

Based on experiments with fluorogenic tri- and tetrapeptides, IFN-γ–dependent alterations in the substrate specificity of proteasomes were reported. In the first reports on this subject, the authors observed that proteasomes isolated from IFN-γ–treated cells gained about twofold higher chymotrypsin-like (Suc-LLVY-MCA–hydrolyzing) and trypsin-like (Boc-LLR-MCA–hydrolyzing) activities (49, 50), but lost about half of the postglutamyl (Clz-LLE-MNA–hydrolyzing) activity (49). The authors suggested that these functional alterations should favor the degradation of proteins to peptides that terminate in hydrophobic and basic residues that are usually found bound to MHC class I. These results were confirmed by some (51), but not by others (18, 52, 53). Studies using polypeptide substrates have yielded results inconsistent with each other and with that obtained with fluorogenic substrates (18, 41). Taken together, the functional consequences of the incorporation of IFN-γ–inducible β subunits remain incompletely understood.

Using as substrates 22- and 24-mer polypeptide sequences corresponding to natural proteins, we show that the characteristic P$_1$ specificity spectrum of proteasomes remains qualitatively unchanged in proteasomes isolated from IFN-γ–treated cells. However, as shown for the first time with polypeptide substrates, we see enhanced hydrolysis of individual peptide bonds with hydrophobic P$_1$ residues by proteasomes of IFN-γ–treated cells compared to that of untreated cells. In addition, we see reduced cleavage of neighboring peptide bonds with acidic P$_1$ residues. Thus, the data presented here for polypeptide substrates agree with that first reported by Gaczynska et al. with short fluorogenic substrates (49). However, together with our results, the rather mild defects of mice genetically deficient in LMP2 or LMP7 (54, 55), as well as the restoration of antigen presentation in LMP2/LMP7/TAP triple-deficient T2 cells by transfection with TAP alone (56–59), argue against drastic qualitative alterations in the cleavage preferences of proteasomes by IFN-γ–inducible β subunits.

Another IFN-γ–inducible element is the enhancer PA28. Recently, Dick et al. reported for the degradation of 19–25-mer substrates by 20S proteasomes, a substantially enhanced rate of accumulation of dual cleavage products by addition of PA28 (34). Here we show that recombinant PA28α is sufficient to induce this effect. This is in line with the recent finding that transfection of PA28α is sufficient to improve the recognition of virus-infected cells by CTLs (60). However, with and without PA28, the same cleavage sites are used and the same products are generated. Thus, the highly conserved general cleavage specificity of the 20S proteasome remains unchanged in the presence of PA28. Nevertheless, PA28 might have been evolved to optimize the capacity of the 20S proteasome for oligopeptide generation, in particular from short substrates.

Evolutionary relationships have been invoked between MHC class I and the hsp70 family of chaperones (61, 62). However, recent structural studies (63, 64) indicate that the homologies between the peptide-binding regions of hsp70 and MHC molecules are less than originally anticipated. Observations suggesting evolutionary links between proteasomes, MHC, TAP, and perhaps hsp70 were recently reported by Kasahara et al. (65) and Katsanis et al. (66). Both the human and the mouse genomes contain three regions with striking homology to the MHC complex, as they com-

prise genes coding for proteasome β-type subunits, ABC transporters, hsp70, NOTCH, and complement components. One of these regions, in addition, harbors the gene for CD1. They speculate that the MHC complex and these homologous regions might have been generated by duplication of an ancestral syntenic group in jawless fish, i.e., before emergence of T cell recognition. This implies that MHC class I–like molecules, and perhaps also TAP, may have existed before the adaptive immune system.

Due to their role in the degradation of unfolded polypeptides, proteasomes are adapted to cleave hydrophobic sequences from the inside of proteins. Most of the abundant self-peptides eluted from MHC class I are derived from highly conserved hydrophobic regions of a restricted set of evolutionary conserved ubiquitous intracellular proteins (67). In view of the possible existence of MHC class I before T cell recognition, it is conceivable that the functional cooperation between proteasomes and the precursor of MHC class I was originally designed to present self-peptides and to serve a purpose other than self–nonself discrimination, for example, inhibition of NK killing. MHC polymorphism and T cell recognition may have evolved subsequently, thus accommodating the greater variety of foreign peptides.

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