Effects of major-histocompatibility-complex-encoded subunits on the peptidase and proteolytic activities of human 20S proteasomes

Cleavage of proteins and antigenic peptides

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The proteasome is responsible for the non-lysosomal degradation of misfolded, transient, or ubiquitin-tagged proteins. This fact and the identification of two major-histocompatibility-complex-(MHC)-encoded proteosomal subunits, LMP2/7, suggest an important role of the proteasome in antigen processing. Using purified 20S proteasomes from a wild-type and a LMP2/7-deletion T lymphoblastoid cell line, we analyzed the effect of LMP2/7 on the peptidase and proteolytic activities of the complex in the context of various purification and activation methods. The incorporation of LMP2/7 alters the peptidase activity against fluorogenic substrates, but these effects are not reflected in the time-dependent degradation pattern of oxidized insulin B chain or of peptide epitopes of an antigenic protein. No effect of LMP2/7 on the degradation pattern of these substrates was observed by either reverse-phase chromatography, pool sequencing, or mass spectrometry. The 20S proteasome can cleave insulin B chain at nearly every position, showing that the P1 position alone does not determine the cleavage sites. The maximum of the length distribution of the end products, makes these ideal candidates for MHC display; yet we find that a natural epitope derived from human histone H3 is further degraded by 20S proteasomes. Alanine scans and substitutions with related amino acids of this epitope indicate that, as in insulin B chain, the cleavage sites are not determined by the P1 position alone.

Keywords: antigen processing; interferon; major histocompatibility complex; multicatalytic proteinase; protein degradation.

The 20S proteasome is a high-molecular-mass (700 kDa) multicatalytic proteinase composed of four stacked, seven-membered rings, that form a barrel-shaped complex (Hegerl et al., 1991; Pührer et al., 1992) found in the cytoplasm and nucleus of all eucaryotic cells (Rivett, 1989; Orlowski, 1990; Driscoll and Finley, 1992). The eucaryotic particle is assembled of at least 14 non-identical, but related subunits of 21–31 kDa (Orlowski, 1990). Recently, the structure and the catalytic center of the Thermoplasma 20S proteasome were solved (Lowe et al., 1995; Seemüller et al., 1995). The purified eucaryotic 20S proteasome exhibits at least three different peptidase activities against short fluorogenic peptides. They were classified as chymotrypsin-like (cleavage at the carboxyl site of hydrophobic amino acids), trypsin-like (cleavage at the carboxyl site of basic amino acids) and peptidyl-glutamyl peptide-hydrolyzing (PGPH; cleavage at the carboxyl site of glutamic acid) activity (Orlowski, 1990; Orlowski et al., 1990; Rivett, 1993). In addition, a further catalytic activity, classified as caseinolytic, is postulated to be responsible for protein degradation (Yu et al., 1991; Mykles and Haire, 1991). Depending on the purification method, proteasomes can be purified in an active or a latent form (Tanaka et al., 1986; McGuire et al., 1989). While the active form is able to degrade proteins, latent proteasomes show only low proteolytic activity which can be activated by different treatments, e.g. SDS, polylysine (Tanaka et al., 1986), cardiolipin (Ruiz et al., 1993), heat (Mykles, 1989) or dialysis against water (McGuire et al., 1989).

The 20S proteasome plays a major role in non-lysosomal degradative processes (Fujisawa et al., 1990; Heinemeyer et al., 1991; Mason and Rivett, 1994; Rock et al., 1994) and as the proteolytic core of the 26S complex in the ubiquitin-dependent pathway (Goldberg, 1992; Hershko and Ciechanover, 1992; Rechsteiner et al., 1993). 20S/26S complexes are postulated to be important for the generation of antigenic peptides (Goldberg and Rock, 1992; Monaco, 1992; Michalek et al., 1993; Rock et al., 1994). These endogenous peptides, which are derived from antigens in the cytosol or nucleus, are transported by the major-histocompatibility-complex-(MHC)-encoded transporter (TAP) into the endoplasmic reticulum (ER), where they associate with MHC class I molecules. On the cell surface, MHC class I molecules present these peptides to cytotoxic CD8+ T cells to initiate the cytotoxic immune response (for review see Yewdell and Bennink, 1992).

The genes of two proteasomal subunits LMP2 and LMP7 (LMP, low-molecular-mass protein) are encoded within the MHC class II region, in close proximity to the tap1 and tap2
Recently, several groups reported that LMP2/7 influence the peptidase activities towards short fluorogenic substrates and that the composition of newly synthesized 20s proteasomes is modified by the interleukin(IFN)-γ-induced exchange of the subunits δ and ε to LMP7 and LMP2, respectively (Yang et al., 1992; Akiyama et al., 1994a,b; Früh et al., 1994; Belich et al., 1994). Recently, several groups reported that LMP2/7 influence the peptidase activities towards short fluorogenic substrates and that the composition of newly synthesized 20s proteasomes is modified by the interleukin(IFN)-γ-induced exchange of the subunits δ and ε to LMP7 and LMP2, respectively (Yang et al., 1992; Akiyama et al., 1994a,b; Früh et al., 1994; Belich et al., 1994). Because of these results, several authors conclude that the expression of LMP2 and LMP7 results in a changed cleavage pattern of proteins without considering that the proteolytic activity should be distinct from the peptidase activities against artificial substrates. According to this hypothesis, the increased chymotrypsin-like and trypsin-like activity should lead to more peptides with a basic or hydrophobic C-terminus and therefore to an increased amount of MHC-restricted epitopes. Until now only a few groups have studied protein degradation by 20S proteasomes (Rivett, 1985; Dick et al., 1991, 1994; Takahashi et al., 1993; Wenzel et al., 1994), consequently, it is not known whether conclusions from the peptidase can be applied to the proteolytic activity.

In this study, we describe the effect of LMP2/7 on various peptidase activities against short fluorogenic peptides in the context of, different purification methods for the 20S proteasome, the state of the 20S proteasomes (latent or active), and the method of activation. Although we determined changed peptidase activities of 20S proteasomes containing LMP2 and LMP7, the proteolytic activity against the oxidized insulin B chain and against a peptide epitope was not modified by the expression of the MHC-encoded subunits. Furthermore, the resistance to proteolysis of an epitope is not altered by the presence of LMP2 and LMP7. The cleavage pattern of the epitope was not influenced by modification with alanine or related amino acids, leading us to the conclusion that a cleavage site is not determined by the P1 position, but, in a more diffuse way, by the entire peptide sequence.

**Materials and Methods**

**Cells and culture conditions.** The lymphoblastoid cell lines T1 and T2 (Salter and Cresswell, 1986) were grown as suspension with RPMI 1640 Glutamax I containing 10% fetal calf serum, 100 U/ml Penicillin, and 100 μg/ml Streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air. Media and supplements were obtained from Gibco. At a cell density of 4×10⁵ cells/ml, 400 U/ml of IFN-γ (Boehringer Mannheim) were added and the culture was continued for 3 days. The cells were harvested by centrifugation, briefly washed with ice-cold NaCl/P, (10 mM sodium phosphate, 140 mM NaCl, pH 7.8) and stored at -20°C.

**Antibodies.** The hybridoma mAb MCP21 specific for the α-type subunit HC 3 was kindly provided and described in detail by Klavd Hendil (Hendil and Uerkvitz, 1991; Kaltoft et al., 1992; Hendil et al., 1995). To produce polyclonal antibodies against LMP2 and LMP7 rabbits were immunized with peptides derived from the C-termini of LMP2 (CHRVLGNELPKFYDE) and LMP7 (CTDVSDLHQQREANQ). Alternatively, LMP2 and LMP7 were expressed in Escherichia coli as histidine fusion proteins and isolated by immobilized-metal-ion chromatography (B. Ehring and R. Tampé, unpublished results). The purified fusion proteins were also used to raise polyclonal antibodies in rabbits.

**Synthesis of peptides.** The peptides in this study were synthesized on a multiple peptide synthesizer (MultiSynTech) by conventional fluorenylmethyloxycarbonyl chemistry. The identity of the peptides was established by mass spectrometry and the purity was determined by reverse-phase HPLC. Purity was at least 93% for each peptide.

**Isolation of 20S proteasomes.** T1 and T2 cells were lysed by ultrasonication (4×15 s) in 20 mM Tris/HCl, 1 mM EDTA, 250 mM sucrose, pH 7.8. The cell lysate was centrifuged at 80000 g for 1 h to remove cell membranes, nuclei and debris. The centrifugate was used for two alternative isolation methods of the 20S proteasome. Method 1. The supernatant was applied to a DEAE-Sephasel ion-exchange column (10 cm×3.5 cm, Pharmacia, Germany) with a flow rate of 0.3 ml/min, washed with TE buffer (20 mM Tris/HCl, 1 mM EDTA, pH 7.8), and eluted at a flow rate of 0.7 ml/min with 400 ml of a linear gradient from 0 to 600 mM NaCl in TE buffer. Using Suc-Leu-Leu-Val-Tyr-NHMe (Suc, succinyl; -NHMe, amidomethyl-coumarin) as a substrate for the chymotryptic activity, fractions with the major activity were pooled after each isolation step. The 20S proteasomes eluted at 340–360 mM NaCl. Active fractions were concentrated by Centriprep 100 (Amicon) and loaded onto a Sephacryl S 300 gel-filtration column (90 cm×2.5 cm; Pharmacia). The proteins were eluted with TE buffer at a flow rate of 0.7 ml/min. Active fractions were subjected to a Mono Q ion-exchange column (Pharmacia) and eluted at 50 ml with a linear gradient from 0 to 500 mM NaCl in TE buffer. The 20S proteasomes found in fractions at 500 mM NaCl were concentrated with Centricon 100 (Amicon) and applied to a Superose 6 gel-filtration column (Pharmacia). Purified 20S proteasomes were eluted at a molecular mass of 600–700 kDa.

Method 2. Proteasomes were isolated by affinity chromatography as described (Hendil and Uerkvitz, 1991). 1.5 mg purified mAb MCP21 was coupled to a 1 ml N-hydroxysuccinimide-activated HiTrap column (Pharmacia) according to the instructions of the manufacturer. Separate columns were used for each cell line. The centrifugate was reincubated over the column for 4 h with a flow rate of 0.2–0.3 ml/min. Unspecifically bound protein was removed by extensively washing with TE buffer, followed by TE buffer containing 50 mM and 200 mM NaCl. 20S proteasomes were eluted with TE buffer containing 2 M NaCl and immediately dialyzed against TE buffer. All procedures of both protocols were strictly performed at 4°C. If not indicated, the affinity chromatography was used for the purification, because it took only one day in contrast to four days for a conventional purification. Protein concentration was determined by the method of Bradford (Bradford, 1976) using BSA as standard. SDS/PAGE was performed as described by Laemmli (Laemmli, 1970) using 8–18% gradient gels. The protein was visualized by Coomassie Blue. 20S proteasomes separated on SDS/PAGE were electrophoretically transferred to nitrocellulose membranes using a semidylo blotter (Biorad). Blots were treated with antibodies, and antigen-antibody complexes were developed by enhanced chemoluminescence (Amersham).
Peptidase activity assays. To analyze the peptidase activity of the proteasomes the fluorogenic substrates (Bachem) Succinyl-LLVY-7-amido-4-methylcoumarin (Suc-LLVY-NHMec; chymotrypsin-like), butyloxycarbonyl-LRR-NHMec (Boc-LRR-NHMec; trypsin-like), benzyloxycarbonyl-LLE-β-naphthylamine (Z-LLE-βNA; peptidyl-glutamyl peptide-hydrolyzing) were used. Varying concentrations of the substrates were incubated with 200 ng (2.9 nM) purified 20s proteasomes in the presence or absence of 0.03% SDS (by mass) at 37°C in 100 μl TE buffer. The reaction was stopped with 500 μl stop solution (100 mM monochloroacetic acid, 130 mM NaOH, 100 mM acetic acid). The concentration of the liberated fluorophore was determined at λex/λem = 360/460 nm in the case of NHMec and λex/λem = 335/410 nm in the case of β-naphthylamine.

Digestion of oxidized insulin B chain and peptide epitopes. 0.5 mg of the oxidized insulin B chain or the antigenic peptides were incubated with 40 μg (57 nM) purified 20s proteasomes in presence or absence of 0.03% SDS (by mass) in 1 ml TE buffer at 37°C. At various time points, the degradation was stopped by addition of formic acid yielding a final concentration of 0.05% (by vol.). The degradation products in the assay mixture were applied to HPLC, liquid chromatography (LC)/MS and pool sequencing.

Reverse-phase HPLC. Chromatography was performed on an HPLC system pump 420, autosampler 360, DAD 440 (Kontron Instruments) and a fluorescence detector RF-535 (Shimadzu). The degradation products of the oxidized insulin B chain or the peptide epitopes were separated on a C18 reverse-phase column (LiChroCart 125-4, Merck). Water (solvent A) and acetonitrile (Merck; solvent B) supplemented with 0.1% trifluoroacetic acid (Sigma) were used as solvent system. The peptide mixture was separated by a gradient from 0 to 50% B over 40 min and a gradient from 50% to 90% over 15 min (1 ml/min). The degradation products were detected at 206 nm, 216 nm and 280 nm. Aromatic residues were identified at λex/λem = 280 nm/340 nm by the fluorescence detector.

Pool sequence analysis of the degradation products. For determination of the major cleavage sites of the insulin B chain, degradation products at different time points were analyzed by sequential Edmann degradation on a gas-phase sequencer 477A (both Applied Biosystems) according to the instructions of the manufacturer. The intensity of cleavage was estimated from the relative yields of amino acids.

Liquid chromatography combined with mass spectrometry. Mass spectrometry was performed on line (LC/MS) by coupling of the SMART-System (Pharmacia) to an atmospheric pressure ionization source (ion spray) fitted to tandem quadrupole instrument API III (Sciex). The peptides were separated on a LiChroCart 125-2 (Merck) and the solvent system was the same as described above. The gradient from 0 to 60% B was generated over 150 min and from 60% to 90% B over 50 min with a flow rate of 20 μl/min. Each scan was acquired over a range m/z 350–2000 using a step of 0.2 and a dwell time of 0.5 ms. The molecular masses of the peptides were obtained as single ions or calculated from the m/z peaks in the charge-distribution profiles of the multiply charged ions (Covey et al., 1988; Mann et al., 1989). Amino acids or dipeptides cannot be detected by this method.

RESULTS

IFN-γ increases the level of LMP2/7 in the purified 20s proteasome. The 20s proteasome complex was purified from two human lymphoblastoid cell lines (T1 and T2) by means of either four-step standard chromatography (anion exchange and size exclusion) or one-step affinity chromatography. Both purification protocols yielded T1 and T2 20s proteasomes with a purity greater than 95%. Beside the typical proteasomal subunit pattern (21–31 kDa), no other protein bands including activators, inhibitors or components of the 26S complex were visible in the SDS/polyacrylamide gel (Fig. 1a). The activity of the 20s proteasome from both preparations differs, although the subunit pattern is identical. Proteasomes purified by conventional chromatography are activated, whereas affinity-purified proteasomes are in a latent state, although no glycerol was used during the entire isolation procedure. These latent proteasomes could be activated by SDS, cardioliopin or other treatments.

The composition of newly synthesized 20S proteasomes is altered in T1 cells by stimulation with IFN-γ, proteasomal subunits δ and ε being substituted by the MHC-encoded LMP7 and LMP2, respectively. In 20S proteasomes of non-stimulated T1 cells, LMP7, but only very small amounts of LMP2, were detected by immunoblotting (Fig. 1b). After stimulation, the amount of LMP2 is significantly increased, whereas the level of LMP7 is only slightly raised. The IFN-γ-induced expression of LMP2 as the smallest proteasomal subunit is directly visible in the MHC class I1 region, the T2 cell line is unable to express LMP2 or LMP7 and therefore the levels of δ and ε are not changed by IFN-γ stimulation. To study the effect of LMP2/7...
on proteasome function, purified 20S proteasomes of IFN-γ treated T1 and T2 cells were compared. Since both cell lines were treated in the same way, effects of IFN-γ-inducible activators or 20S proteasome subunits (e.g. MECL-1, Tanaka, 1995) are identical and do not influence the comparison of T1 and T2 20S proteasomes. No difference in the overall structure of T1 and T2 20S proteasomes was observed by high-resolution electron microscopy, independently of the preparation method.

**LMP2 and LMP7 changes the peptidase activity of the 20S proteasome against artificial substrates.** Because conflicting results have been reported for the influence of IFN-γ or LMP2/7 on the peptidase activities against various artificial substrates, we examined this problem more closely in the context of different isolation methods, purity, and the active state of the 20S proteasome. Therefore we analyzed the enzyme kinetics of IFN-γ-treated T1 and T2 20S proteasomes in an activated and latent state with three substrates (Suc-LLVY-NHMec, Boc-LRR-NHMec, Z-LLE-βNA). Unlike other studies that used clarified crude extracts or partially purified proteins, this study was performed entirely with highly purified proteasomes. In consequence, the interference of any other protein such as activators or inhibitors is highly unlikely, and the enzyme activities measured are directly related to a pure enzyme complex.
The chymotrypsin-like activity (Fig. 2a,b) and the hydrolysis of Boc-LRR-NHMec by latent 20S proteasomes (Fig. 2c) follows Michaelis-Menten kinetics. The peptidyl-glutamyl peptide-hydrolyzing activity measured with the substrate Z-LLE-βNA is biphasic for latent 20S proteasomes, but not for activated 20S proteasomes (Fig. 2c,f) in accordance with previous results (Orlowski et al., 1991; Djaballah and Rivett, 1992). The $V_{\text{max}}$ and $K_{\text{m}}$ values of T1 and T2 20S proteasomes for different substrates are summarized in Table 1. In the latent state, peptidase activities of T1 20S proteasomes against all three tested fluorogenic peptides are increased relative to T2 20S proteasomes, but the trypsin-like activity clearly more than the PGHP-like and chymotrypsin-like activities, which are only slightly increased. In the active state, the chymotrypsin-like activity of T1 20S proteasomes is increased, the trypsin-like activity is identical and the PGHP-like activity is slightly decreased relative to T2 20S proteasomes. As shown, none of the activities of the 20S proteasomes is the same in the latent and active forms. In summary, the treatment with 0.03% SDS resulted in changes of all three peptidase activities, as had been reported previously (Ugai et al., 1993; Aki et al., 1994). The cleavage rate as well as the substrate affinity are changed by the activation state of the 20S proteasome. The activation during purification or in the presence of cardiolipin has similar effects on the cleavage rates and substrate affinities for T1 and T2 20S proteasomes (data not shown). In the case of activated 20S proteasomes, our results confirm that the incorporation of LMP2 and LMP7 stimulates cleavage after hydrophobic residues (P1 position) and suppresses cleavage after acidic residues, but the effects are far from being dramatic and depend on the activation state and on the purification method.
LMP2/7 do not influence the time-dependent degradation of oxidized insulin B chain. The natural function of the proteasome complex is to degrade misfolded, modified, or short-living proteins. Therefore, instead of using fluorogenic substrates, we decided to analyze the degradation of oxidized insulin B chain, which has been already used as a model substrate for the 20S proteasome (Rivett, 1985; Dick et al., 1991; Takahashi et al., 1993; Wenzel et al., 1994). Here, we address the basic question of whether the influence of LMP2 and LMP7 on the peptidase activity against artificial substrates is also reflected in the time-dependent degradation of a protein fragment of 30 amino acids containing 9 hydrophobic, 7 basic and 2 acidic cleavage sites. If the proteolytic activity is altered in the same way as the peptidase activity, a changed cleavage pattern should be expected. That 20S proteasomes in a latent state degrade oxidized insulin B chain only very slowly gave us a first hint that this substrate is cleaved by the proteolytic activity and not by various peptidase activities.

We compared the cleavage pattern of IFN-γ-stimulated T1 and T2 20S proteasomes at up to 10 different time points, which encompassed the formation of the very first fragments, transient products, and end products. The products were analyzed by reverse-phase HPLC, pool sequencing, and mass spectrometry. Examples at different time points (0.25, 1, 4 h) are given in Fig. 3. After 4 h, the degradation by activated proteasomes was completed and prolonged incubation or further addition of proteasomes did not alter the cleavage pattern. Obviously, these peptides are end products and resistant against further digestion. Furthermore, even an eightfold increase of the substrate concentration did not change the cleavage pattern. Since none of the peaks was present in assays without enzyme or substrate, all end products are derived from the substrate. Protein degradation was
Fig. 3. Digestion products of the oxidized insulin B chain at various time points were analyzed by reverse-phase HPLC. 0.5 mg of the oxidized insulin B chain was incubated with 40 μg purified IFN-γ-stimulated T1 or T2 20S proteasomes in 1 ml TE, pH 7.8, at 37°C. 20S proteasomes were activated by 0.03% SDS. After 15 min, 60 min and 4 h, 50-μl samples were taken, the reaction was stopped with 0.05% formic acid and applied to the HPLC. The degradation products were detected by a diode array detector. Chromatograms at 206 nm are given.

Table 1. The $V_{\text{max}}$ and $K_m$ values for the peptidase activities of IFN-γ-stimulated T1 and T2 20S proteasomes in the latent and SDS-activated (in parentheses) form. The values for the chymotryptic activity of latent and activated 20S proteasomes as well as for the trypsinic activity of latent 20S proteasomes were analyzed by Michaelis Menten kinetics. The $V_{\text{max}}$ and $K_m$ values for the trypsinic activity of activated 20S proteasomes could not be determined, because the substrate is not soluble above 250 μM in the presence of 0.03% SDS. The $V_{\text{max}}$ and $K_m$ values for the PGHP activity were estimated from Fig. 2e, f.

<table>
<thead>
<tr>
<th>Activities</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
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<tbody>
<tr>
<td>Trypsin-like activity of T1</td>
<td>2594</td>
<td>551</td>
</tr>
<tr>
<td>Trypsin-like activity of T2</td>
<td>671</td>
<td>271</td>
</tr>
<tr>
<td>Chymotrypsin-like activity of T1</td>
<td>1045 (8632)</td>
<td>155 (38)</td>
</tr>
<tr>
<td>Chymotrypsin-like activity of T2</td>
<td>812 (3373)</td>
<td>182 (16)</td>
</tr>
<tr>
<td>PGHP activity of T1</td>
<td>420 (4940)</td>
<td>139 (89)</td>
</tr>
<tr>
<td>PGHP activity of T2</td>
<td>295 (6175)</td>
<td>132 (76)</td>
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</table>

Fig. 4. Major cleavage sites of the oxidized insulin B chain used by IFN-γ-stimulated T1 and T2 20S proteasomes identified by pool sequencing. The large or small arrows indicate cleavage sites preferentially used at early or late stages of digestion, respectively. C represents the oxidized form of cysteine.

completely blocked by immunoprecipitation of the 20S proteasome using mAb MCP21, demonstrating that the entire degradation process is proteasome specific.

No difference in the cleavage patterns between activated T1 and T2 20S proteasomes was observed at various time points (Fig. 3). The time-dependent degradation of the oxidized insulin B chain was fitted by a single exponential function with a half-life of 19.3 min for T1 and 18.6 min for T2 20S proteasomes. In addition, the degradation profiles of non-stimulated T1 and T2 20S proteasomes were identical (data not shown). To exclude artifacts related to the presence of SDS, we also compared the degradation by latent 20S proteasomes including prolonged incubation periods (up to 120 h), by 20S proteasomes activated with cardiolipin, heat, or during conventional purification without further treatment. In all cases the HPLC chromatograms showed nearly the same pattern (data not shown). Due to the purification and activation methods the cleavage rate differs, but IFN-γ-stimulated T1 and T2 20S proteasomes treated with one method always had the same cleavage rate. To eliminate the possibility that for some unknown reasons the 20S proteasomes of the mutant T2 cell line show an abnormal behaviour in proteolysis, 20S proteasomes isolated from an IFN-γ-stimulated renal carcinoma cell line (MZ1257ERC; Seliger, B., unpublished results) were also used. The digestion yielded the same time-dependent degradation pattern (Ehring, B. and Tampt, R., unpublished results).

We analyzed product formation by HPLC, since this is the optimal method to observe any qualitative as well as quantitative differences in the protein-cleavage pattern. To avoid potential problems with very hydrophobic fragments, we also determined the main cleavage sites by pool sequence analysis at different time points of the digestion (Fig. 4). An important feature of the degradation of the oxidized insulin B chain is that the cleavage pattern is changed over time. Some peptides are transient, they appear at the beginning and disappear over time. Other peptides are generated only at a later stage of proteolysis. The pool sequence analysis confirmed the results obtained by HPLC analysis that 20S proteasomes with and without LMP2 and LMP7
Fig. 5. Identified peptide fragments of the oxidized insulin B chain generated by IFN-γ-stimulated T1 or T2 20S proteasomes after 15 min (a) and 4 h (b). The degradation assay was the same as in Fig. 4. The peptides were analyzed by LC/MS. Some masses cannot directly be assigned to one peptide. Fragments marked with (*) and a number have the same molecular mass. Length distribution of the identified peptide fragments of the oxidized insulin B chain by T1 and T2 20S proteasomes after 15 min (c) and 4 h (d). Note that the histogram does not reflect the amount of each peptide.
produce an identical cleavage pattern and use same cleavage sites with the same frequency. As mentioned before, there are differences in the degradation pattern at early and later stages of digestion. All major cleavage sites identified by pool sequencing are used from the beginning, but to different extents. As indicated in Fig. 4, 20S proteasomes cleaved some positions preferentially at later stages of proteolysis.

20S proteasomes can cleave insulin B chain at nearly every position, leading to a time-dependent length distribution of the fragments. We next concentrated on the question of which fragments are generally produced by the 20S proteasomes without considering the amounts produced. To this end, the time-dependent degradation of oxidized insulin B chain was analyzed by LC/MS (Fig. 5). At early (15 min) and late stages of the digestion (4 h) the identified cleavage products are quite different. The important characteristics of the fragmentation at early time points are: many peptides correspond to the N-terminus or C-terminus of the oxidized insulin B chain, which are generated by a single cleavage and then released; in addition to the most frequent cleavage sites identified by pool sequencing, the substrate is cleaved at almost every peptide bond; and the distribution of the peptide length is broad and varies in the range 4-27 amino acids. In contrast, at the end of the digestion only a few end products possess the original N-terminus or C-terminus of the substrate; most of the longer peptides at the early stages disappear, which means they are cleaved again; and the length distribution of end products is narrow with a maximum at 7-10 amino acids. Thus, the peptide composition and especially the peptide-length distribution changes drastically during the process of degradation. Note that single amino acids and dipeptides cannot be detected by this method.

Cleavage pattern and stability of peptide epitopes. The time-dependent degradation of the histone H3 epitope (RRYQKS-TEL) by activated IFN-γ-stimulated T1 or T2 20S proteasomes was compared by HPLC and mass spectroscopy. The recognition of cleavage sites was studied using peptides modified by alanine or related amino acids. These peptides have already been used to analyze peptide selection to the MHC-encoded transporter complex (TAP; Uebel et al., 1995). Here, we found that the original epitope and the related peptides are not resistant against degradation by the 20S proteasome. The degradation rates of all tested peptides were identical. It can be concluded that the sequence of the epitope is not the crucial factor in preventing its degradation, and the presence of LMP217 neither protects the epitope from degradation nor alters the degradation pattern (Fig. 6). The digestion of the peptides by latent 20S proteasomes is very slow, indicating that again the proteolytic and not the peptidase activity is responsible for the degradation, although it is only a nine-residue peptide.

Next, we were interested in whether distinct amino acid substitutions in the epitope will influence the cleavage pattern. Therefore, fragments of individual HPLC peaks were collected, concentrated, and identified by MS. All analyzed peptides were cleaved at the same positions. The cleavage pattern of 13 tested peptides is summarized in Fig. 7. At early stages of the degradation, position 3 was identified as the major and position 4 as a minor cleavage site, resulting in the fragments 1–3 and 4–9, and 1–4 and 5–9, respectively. At later stages, fragment 5–9 was partially degraded into fragments 6–9 and 7–9. A further cleavage yielding the dipeptide 8–9 could not be determined by the methods used (HPLC and MS). We could also not determine whether fragment 4–9 was cleaved again. In addition, some smaller peaks in the HPLC chromatograms derived from limited hydrolysis of the peptide bonds after position 1 or 2 were identified. The cleavage at all these positions was not affected by substitutions at the cleavage site or at any other position by alanine or related amino acids. Also the cleavage rate of the sub-
Histone H3 epitope

\[
\text{RRYQKSTEL} \\
\text{RRYASTEL} \\
\text{RRASTEL} \\
\text{RRASTEL} \\
\text{RRYASTEL} \\
\text{RRASTEL} \\
\text{RRYASTEL} \\
\text{RRYASTEL} \\
\text{RRYASTEL}
\]

\[
1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8 \quad 9
\]

Fig. 7. Cleavage pattern of the epitope and modified peptides derived from the human histone H3. The substitution of single residues are grey shaded. The digestion assay was performed as indicated in Fig. 6. Cleavage products were separated by reverse-phase HPLC and identified by mass spectrometry. The numbers 1–9 represent the position of each amino acid in the peptide chain.

The substitution of single residues is nearly identical for all peptides (data not shown). In a few cases, the cleavage ratio at position 3 and position 4 was slightly changed in the early stages of proteolysis.

DISCUSSION

The role of LMP2/7 in the peptidase activity of the 20S proteasomes. IFN-γ-stimulation results in an incorporation of LMP2 and LMP7 into 20S proteasomes, although low levels of these subunits can be detected in constitutively expressed proteasomes. To analyze the effect of LMP2 and LMP7 on the peptidase and proteolytic activities, it is therefore most useful to compare isolates from a wild-type (T1) and a mutant cell line (T2), which is genetically deleted for LMP2 and LMP7. Using two cell lines treated in the same way has the additional advantage that other effects of the cytokine do not influence the comparison. Finally, highly purified preparations must be used, since, in partially purified samples, an IFN-γ-inducible activator (Realini et al., 1994) can lead to changed peptidase activities (Ustrell et al., 1995).

Our results demonstrate that the incorporation of LMP2 and LMP7 alters all three peptidase activities of latent and also the chymotrypsin-like and PGHP activity of activated 20S proteasomes. The trypsin-like activity of activated 20S proteasomes is not affected by the expression of LMP2/7 which was also found for proteasomes of a human renal carcinoma cell line (Aki et al., 1994). This is the expected result, considering that the yeast homologues of LMP2 and LMP7 (PRE3 and PRE2) are responsible for the PGHP and chymotrypsin-like activities of the yeast proteasome complex (Enenkel et al., 1994; Heinemeyer et al., 1993). The chymotrypsin-like activity of latent and activated, as well as the trypsin-like activity, of latent 20S proteasomes is increased by the expression of LMP2/7. However, the PGHP activity of latent 20S proteasomes is also higher for cells expressing the MHC-encoded subunits, while PGHP activity of activated 20S proteasomes is slightly lower than for the mutant cells. With respect to the effects of MHC-encoded subunits our results agree with recently published results (Gaczynska et al., 1993, 1994; Driscoll et al., 1993; Aki et al., 1994; van Kaer et al., 1994), but are different from results reported by two other groups (Boes et al., 1994; Ustrell et al., 1995).

Although it is clear that the peptidase activities measured with short fluorogenic peptides are modified by LMP2/7, the magnitude of the effect is small and difficult to quantify, \( K_m \) and \( V_{\text{max}} \) values changing by only a factor of 1.2–5. In addition, the 20S proteasomes of one cell line do not have the same peptidase activities after different methods of isolation or after activation by SDS, heat or cardiolipin. An important reason for the difficulty in comparing the influence of LMP2/7 on the peptidase activities, is that isolation methods, purity and state of the 20S proteasome vary from group to group.

20S proteasome-mediated degradation of polypeptides. The effects of LMP2/7 have been characterized by using artificial fluorogenic substrates. An important aim of this study was to investigate whether the influence on the peptidase activity against fluorogenic substrates is also reflected in a change of the cleavage pattern of peptide epitopes or short proteins. The oxidized insulin B chain was chosen as model substrate, since its digestion by eucaryotic proteasomes has already been investigated (Rivett, 1985; Dick et al., 1991; Takahashi et al., 1993). Detailed analysis of the degradation by IFN-γ-stimulated T1 and T2 20S proteasomes at various time points demonstrates that the incorporation of LMP2/7 does not influence the degradation of this substrate. Kinetics of degradation as well as the cleavage pattern of the oxidized insulin B chain at different time points are nearly identical. Nevertheless, because antigenic epitopes were found to be produced at very low amounts (less than 1 mol/100 mol of the total protein; Dick et al., 1991), we cannot exclude the possibility that subtle effects on the peptidase activities (maximum 1.2–5-fold), undetectable by reverse-phase chromatography, pool sequencing, or mass spectrometry, are important in the cellular context. Cytotoxic T-cell assays would be the only method sensitive enough to see differences in the generation of known epitopes, but in these assays, unknown mechanisms for epitope generation can cause misleading effects.

Major cleavage sites in the oxidized insulin B chain were identified by pool sequence analysis. With the exception of the Leu-Cys7 bond, which is not cleaved by the 20S proteasome of T1 and T2 cells, the results are consistent with data from proteasomes of rat liver or human erythrocytes (Rivett, 1985; Dick et al., 1991). Because of this accordance, we abstained from identifying single peaks of the reverse-phase chromatograms. All major cleavage sites determined by pool sequencing can be found even at the beginning of the digestion. We can only distinguish between cleavage sites which were preferentially used earlier or later in digestion. The degradation seems to be a sequential process and later cleavage sites are more accessible for catalytic centers after initial cleavage.

Information about all possible cleavage products at different time points of the digestion was obtained by LC/MS. The identification of all fragments indicates that the enzyme can cleave at almost any amino acid of the polypeptide. A differentiation, as postulated for the peptidase activities, is not permissible for the proteolytic activity towards this polypeptide. There seems to be no restriction at the P1 position and amino acids other than those of the three peptidase activities can represent major cleavage sites of the oxidized insulin B chain. Therefore, simple conclu-
sions from short fluorogenic peptides to polypeptides are not justified. Additional recognition sites of the substrate, which directly proteolysis, must be taken into account.

The second important result of the fragment analysis was the length distribution of the produced peptides. While at the beginning of the digestion the length of the peptides is distributed across a wide range, the length distribution of the end products has a maximum at 7–10 amino acids, which is nearly the size of peptides presented by MHC class I molecules. It was demonstrated that eucaryotic proteasomes are able to generate and accumulate peptides of a length that is appropriate for antigen presentation. A similar length distribution was found for the degradation of the same substrate by the archaeabacterial proteasome (Wenzel et al., 1994), although the cleavage pattern is different to that of various other eucaryotic proteasomes (B. Ehring and R. Tampé, unpublished results). These results fit to the X-ray structure of the Thermoplasma 20S proteasome (Löwe et al., 1995) that a polypeptide can occupy two adjacent active sites (3 nm in distance) in an extended conformation of eight amino acids. Although it appears from these results that the 20S proteasome cleaves antigenic proteins to peptides with a size appropriate for binding to MHC class I molecules and that our immune system recruits an already established machinery of protein breakdown, our experiments cannot eliminate the possibility that a further processing or trimming occurs by other cellular factors.

Generation and stability of antigenic peptide epitopes. It is still an open question, how MHC-restricted epitopes are generated and whether LMP2/7 have any influence on this process. Two questions emerge in the context of antigen processing and hierarchy of epitopes presented by MHC class I molecules. Are dominant epitopes formed, because they are, once produced, more resistant against further digestion by the proteasome? Does LMP2 or LMP7 affect the stability and degradation pattern of peptide epitopes? To answer these important questions, the proteasome-catalyzed degradation of an epitope derived from human histone H3 was analyzed in detail. The epitope and related peptides are not resistant against processing by the 20S proteasome. In addition, no influence of LMP2/7 on the kinetics and degradation pattern of all investigated peptides was observed. Only one group has studied the influence of LMP2/7 on proteolytic activity (Boes et al., 1994). In contrast to our results, they found differences in the cleavage pattern of an extended epitope, although the amount of generated epitope was not influenced by LMP2. We cannot explain this discrepancy, but the peptidase activities determined by this group are quite different from ours or from those of most other groups.

Two controversial hypotheses can be given for the generation of peptide epitopes. From data of the peptidase activity against short fluorogenic substrates some groups conclude that the P1 position determines the cleavage site. It was suggested that an enhanced activity towards hydrophobic and basic amino acids should lead to an increased production of peptide that could bind to MHC class I molecules (Gaczyńska et al., 1993). Since the comparison of a large variety of different epitopes reveals a residue preference for the P1 position at the C-terminal cleavage site, but not at the N-terminal cleavage site, most epitopes cannot be generated directly and further trimming would be indispensable. Our results are not compatible with this hypothesis, since the cleavage sites are not determined by the P1 position.

Flanking sequences of the epitope direct the 20S proteasome to clip the epitope that should be resistant against further degradation (Niedermann et al., 1995). Since in the study of Niedermann et al. (1995), degradation products were analyzed only by pool sequencing and not separated and identified individually by chromatography and MS, answers about the time-dependent degradation, amount and life-time of generated epitopes could not be given. Our data demonstrate that at least in our case, an epitope is not resistant to degradation and that therefore this hypothesis is not generally applicable. In this context, it is interesting to note that several epitopes represent only a minute fraction of all cleavage products of antigenic proteins such as ovalbumin, β-galactosidase, or MCMV pp89 (Dick et al., 1994; Boes et al., 1994). A variant of this hypothesis may nevertheless apply in vivo, if the transiently produced antigenic epitopes are immediately scavenged by the cellular factors, such as chaperones or the transport machinery.

For the first time, cleavage and stability of epitopes and related peptides are investigated in a systematic approach. An identical cleavage pattern for peptides modified by alanine scans or related amino acids was found, demonstrating that the amino acid in P1 position responsible for the classification in chymotryptic, tryptic and PGHP activity is not the crucial factor for the cleavage specificity of natural substrates. In addition, adjacent residues (e.g. P2, P3, P1', P2' and P3') may direct the cleavage. Such effects can never be studied, if artificial substrates with hydrophobic fluorophores at the C-terminus are used. In contrast, the fluorophore and the N-terminal protecting group may contribute to the cleavage specificity. In summary, many residues direct substrate binding and cleavage. The electron density of a peptide aldehyde inhibitor in the X-ray structure of the archaeabacterial proteasome demonstrates that the inhibitor is not only aligned via P1, but also via P2 and P3 position (Löwe et al., 1995). As only an inhibitor of three amino acids was co-crystallized, information about the P1', P2' and P3' is missing. Solving the structure with larger substrates or mutants unable to cleave the pro-sequence of the β-subunit will help us to understand the substrate specificity and recognition mechanism of the proteasome. In a systematic approach, this report underlines the broad, but length-restricted substrate specificity of the eucaryotic proteasome and that one amino acid (P1 position) is not sufficient to determine specificity.

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REFERENCES
