Antitopes Define Preferential Proteasomal Cleavage Site Usage

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Protein degradation by proteasomes is a major source of peptides presented by major histocompatibility complex I proteins. Importantly, interferon-γ-induced immunoproteasomes in many cases strongly enhance the generation of antigenic peptides both in vitro and in vivo. Whether this is due to enhanced substrate turnover or to a change in proteasomal cleavage specificity is, however, largely unresolved. To overcome the problems of peptide quantification inherent to mass spectrometry, we introduced the “antitope” as substrate-specific internal standard. The antitope is a non-functional peptide that is generated by proteasomal cleavage within the epitope, resulting in partial overlaps with the functional epitope. Using antitopes as internal standards we demonstrate that the observed enhanced immunoproteasome-dependent presentation of the bacterial listeriolysin O T-cell epitope LLO(296–304) is indeed due to altered cleavage preferences. This method is also applicable to other major histocompatibility class I epitopes as is shown for two potential epitopes derived from Coxsackievirus.

Proteasomes are multisubunit protease complexes that perform most of the non-lysosomal ATP-dependent proteolysis in eukaryotic cells. The 26S proteasome complex is responsible for the degradation of polyubiquitylated proteins and is formed by the so-called 20S proteasome catalytic core that is capped at one or both ends by the 19S regulatory particles (1, 2). The catalytic 20S core proteasome itself is responsible for processing of denatured non-ubiquitylated proteins or for further processing of longer peptides of different origin. Overall, products of proteasomal cleavage are peptides of 3–20 amino acids in length (3). Among these cleavage products are peptides that fulfill the criteria for binding to MHC2 class I molecules with regard to appropriate length and the correct position of anchor residues. Thus, proteasomes generate the adequate C terminus of most investigated MHC class I ligands, whereas the N terminus is often elongated by two or three residues, requiring N-terminal trimming by amino peptidases to allow efficient binding to MHC class I molecules (4–6).

The 20S proteasome catalytic core is built from 28 subunits arranged as four heptameric rings (7). The two outer rings contain the seven structural α subunits; the two inner rings each contain seven β subunits (β1–β7), of which three (β1, β2, β5) exert catalytic activity. Stimulation of cells with IFN-γ induces the expression of three additional catalytic proteasome subunits. The cytokine-inducible subunits β1i, β2i, and β5i are incorporated into the 20S proteasome core upon its de novo synthesis, thus forming the so-called immunoproteasomes (8, 9). Their incorporation alters the catalytic characteristics of the 20S proteasome core. A large number of studies have shown that 20S proteasomes liberate MHC class I epitopes or their N-terminal-elongated precursors out of large polypeptides or denatured proteins in vitro with rates that correlate well with those of the production of antigenic peptides in intact cells (10–12). Furthermore, detailed studies on the functional importance of immunoproteasomes revealed that in particular the generation and presentation of viral epitopes is strongly enhanced in the presence of immunoproteasomes and that their function is tightly connected with the early phases of an antiviral immune response (13–15). Recently, we also demonstrated that the efficient presentation of the bacterial Listeriolysin O T-cell epitope LLO(296–304) requires the presence of immunoproteasomes (16).

Although the biological relevance of immunoproteasomes for an appropriate cellular immune response gradually emerges, the reason for this at the molecular level is by far less clear. In fact, it appears that, depending on the epitope, immunoproteasomes exert their function quite differently. Immunoproteasomes seem to favor cleavage behind hydrophobic residues, which are the predominant anchor residues required for MHC class I binding of antigenic peptides and thus may explain their positive effect on antigen generation (17, 18). On the other hand, there exist an increasing number of examples demonstrating that immunoproteasomes also can down-regulate epitope production by enhanced cleavage within the epitope, i.e. epitope destruction or less efficient liberation (19, 20). Furthermore, in vitro experiments suggest that immunoproteasomes often degrade substrates considerably faster than their constitutive counterparts. Thus, improved epitope generation...
might not necessarily be the consequence of altered cleavage specificities but may also be the result of enhanced substrate turnover. The latter argument seems to be supported by the finding that there is, in general, little difference in the overall quality of peptides that are generated by either of the two types of proteasomes (18). In consequence, in most in vitro experiments it is difficult to decide whether the observed positive effects of immunoproteasomes on epitope generation are due to an increased substrate turnover or altered cleavage specificity.

Based on mass spectrometry and the analysis of the bacterial Listeriolysin O T-cell epitope LLO(296–317) and of three other epitopes, one self- and two virus-derived epitopes, we here report an experimental approach that takes advantage of a substrate-specific non-functional cleavage product, named antitope, that allows one to determine whether predominant generation of a given epitope is due to enhanced substrate turnover or altered cleavage site preference.

MATERIALS AND METHODS

Proteasome Purification from Infected Mice—20S proteasomes were isolated from tissues of wild-type C57Bl/6 mice as well as of IFN-γ receptor-deficient (IFNγR−/−) mice. Control mice (day 0) or mice infected with 5 × 10^7 Listeria monocytogenes at days 2, 4, or 6 post infection (p.i.) as indicated in the figures were sacrificed and organs were frozen in liquid nitrogen (16). Further purification steps followed as described (21). Because IFNγR−/− mice died after day 4 p.i., organs were taken from mice at days 2 and 4 p.i.

Proteasome Purification from Cells—T2 and T2.27mp cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics under 5% CO2. Proteasomes were isolated from T2 cells for standard proteasomes and from T2.27mp cells that stably express all three immunosubunits for immunoproteasomes. Proteasome isolation was performed as described previously (22). The proteasome was measured at 280 nm and analyzed by SDS-PAGE. The yield was calculated at ~90–95%. Equal amounts of proteasomes in assays were adjusted by densitometry of proteasomes in Coomassie-stained gels. Incorporation of immunosubunits was controlled by two-dimensional PAGE and Coomassie staining for proteasomes isolated from liver and small intestines (16) or for proteasomes isolated from T2 or T2.27 cells by Western blot using specific antibodies (22).

Peptides—Peptides were synthesized using standard Fmoc (N-(9-fluorenylethoxycarbonyl) methodology (0.1 mmol) on an Applied Biosystems 4334A automated synthesizer. The peptides were purified by HPLC and analyzed by mass spectrometry (ABI Voyager DE PRO).

In Vitro Peptide Digest—To determine proteasome-mediated processing of a synthetic model peptide, 3 μg of a 27-mer peptide derived from Listeriolysin O (LLO(291–317)) (AYISSVAYGRQV YLKLSTNSHTKVKVCA) was incubated at 37 °C for 2 and 4 h with 1 μg of isolated 20S proteasomes in 100 μl of buffer containing 20 mM Hepes/KOH, pH 7.8, 2 mM magnesium acetate, 2 mM dithiothreitol. Further, 2.5 μg of peptide derived from murine Hsp60(171–200) (VATISANGDKDIGNIISD AMKKVGRKGVIT) and 3 μg each of peptides derived from Coxsackievirus B3 Nancy (CVB3) core protein P2C(1161–1190) (ATIEQAPSQSDQE QLFSNQVYFAHYCRCY) and RNA-directed RNA polymerase P3D(2158–2185) (RKIRISVPGVR CLTLPAFSTLLRRKWLDSF) that were predicted by the SYFPEITHI data base (23) were incubated with 1 μg of proteasomes at 37 °C for the indicated time. Adding trifluoroacetic acid to a final concentration of 0.3% stopped the reactions.

Peptide Identification, Quantification, and Statistical Analysis—Peptides generated by proteasomes were analyzed by reverse phase HPLC, system HP1100 (Hewlett-Packard) equipped with an RPC C2/C18 SC 2.1/10 column (GE Healthcare). Analysis was performed online with an LCQ ion trap mass spectrometer equipped with an electrospray ion source (ThermoQuest) (21). In Fig. 3, A and B, the kinetic of one representative digest is shown. For analyses of linearity of peptide signals in LC-ESI, different amounts of LLO precursor and epitope peptides were separated on HPLC followed by LC-ESI ion trap MS. For statistical analysis of the LLO and the Hsp60 peptides, ion counts were normalized to the 9GPS standard peptide, which was added in equal amounts to each stopped reaction and the mass of which does not interfere with the masses of any proteasomal cleavage products of analyzed peptides. For LLO(291–317) and Hsp60(171–200) four replicates were averaged each. Statistical significance was attributed to differences in one-tailed, heteroscedastic t-test (p < 0.05) or in the non-parametric Mann-Whitney t-test (p < 0.05). The processing of CVB3-derived peptides P2C(1161–1190) and P3D(2158–2185) was repeated two and three times, respectively.

To determine whether immunoproteasomes display increased cleavage site preference, the generation of indicated antitopes that excludes processing of the correct epitopes was used as reference. Ion counts of the antitope were set as 1 in each reaction, and relative amount of corresponding epitope or precursor to the antitope was calculated (means are shown). The analyzed antitope sequences used are indicated in Figs. 1C, 4A, and 5A.

RESULTS

20S immunoproteasome (i20S proteasomes) -dependent in vitro antigen-processing assays using synthetic polypeptides as substrates containing an MHC class I epitope in many cases exhibit an enhanced liberation of the epitope when compared with that from standard proteasomes (s20S proteasomes). Such an effect could be the result of either an overall faster substrate turnover rate or of altered cleavage site preferences of i20S proteasomes that favor the liberation of the epitopes.

Immunoproteasomes Exhibit a Faster Substrate Turnover Rate—To distinguish between the two above possibilities we used identical amounts of purified s20S and i20S proteasomes for in vitro processing assays and analyzed the turnover rates of LLO-derived 27-mer polypeptides that harbor the LLO(296–304) T-cell epitope. As shown in Fig. 1A, i20S proteasomes isolated from T2 cells that were transfected with immunosubunits (T2.27mp) as well as from liver of Listeria-infected mice reveal a significantly accelerated degradation of the polypeptide substrate. Analysis of substrate turnover by s20S proteasomes from liver of uninfected control mice (WT) and i20S protea-
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C57Bl/6 mice infected with i20S proteasomes isolated from T2.27mp cells (open), by incubation with standard proteasomes isolated from T2 cells (open squares) or isolated from liver of wild type C57Bl/6 mice (open diamonds). The substrate degradation was analyzed by ESI-MS after reverse phase HPLC. Means (± S.E.) of four independent experiments for the indicated proteasomes are shown. B, degradation of LLO(291–317) 27-mer peptide is shown after 2 h of incubation with proteasomes from mice of control wild-type (wt) C57Bl/6 mice (d0), after infection with L. monocytogenes (d2 and d6 p.i.), and with proteasomes isolated from liver of IFNγ-R−/− mice without (d0) and after (d2 and d4 p.i.) infection. Means (± S.E.) of four independent experiments are shown. The significance of differences was calculated by non-parametric Mann-Whitney test; p < 0.05 is indicated (*). C, in the scheme peptides derived from LLO(291–317) degradation identified by ESI-MS/MS are shown. Two potential MHC class I ligands, Ala-7-Leu-14 and Val-6-Leu-14 (*), and the precursor peptide Ser-4-Leu-14 are indicated. The so-called antitope Ser-4-Val-12 created which were only identified in digests with i20S proteasomes, are shown as stripped bars. Main cleavage sites are marked with arrows; the black arrows are responsible for generation of epitopes (MHC class I ligands) and precursors.

To study whether the quality of peptides generated from LLO 27-mer polypeptides differ between s20S and i20S proteasomes, the peptide fragments generated by the two proteasome types were compared (Fig. 1C). No major differences in the quality of peptides generated by either subtype were observed. Only two additional fragments were identified by mass spectrometry in digests with i20S proteasomes. However, both resulted from the usage of cleavage sites that are in common with those of s20S proteasomes. Remarkably, with the cleavage at Val-12 ↓ Tyr-13 we detected a strong cleavage site within the epitope whose usage could be crucial for the amount of epitope or epitope precursor peptides generated (Fig. 1C). The peptide fragment Ser-4-Val-12 resulting from cleavage at Val-12 ↓ Tyr-13 possesses an N-terminal residue (Ser-4) identical to the one of the longest epitope precursor peptide but lacks the correct C terminus of the LLO epitope. We therefore decided to use this peptide fragment (Ser-4-Val-12) as suitable internal standard for the calculation of relative cleavage site preference. Because this peptide fragment precluded the generation of a functional MHC class I epitope it was named antitope.

To determine relative amounts of proteasome-generated peptide fragments by semiquantitative mass spectrometric analysis, an interference of peptides of interest must be excluded. For example, more efficient ionization of one peptide may result in better detection compared with another poorly ionized peptide although identical or even less absolute amounts are generated by the proteasome. Therefore, we first demonstrated the linearity between ion current and peptide amount in the range of up to 200 pmol for the 27-mer substrate, both epitopes (Val-6-Leu-14; Ala-7-Leu-14), the precursor peptide Ser-4-Leu-14, and the antitope Ser-4-Val-12. As shown in Fig. 2, all peptides reveal a peptide concentration-dependent linearity whereby the absolute intensities differ between the peptides. In consequence, an increase in the relative ratio of ESI-MS intensity between two of these peptides will be due to an increase in the relative ratio of respective peptide amounts.

Furthermore, possible interferences between peptides in complex mixtures may quench the signal. Therefore, we analyzed the same peptides in different mixtures containing different ratios of the peptides. Although we observed interferences between the peptides and also a decrease in ion intensities, the mass/signal ratios between the peptides remained the same (data not shown).

Cleavage Site Preference or Degradation Rate—Using the antitope as defined above as internal substrate-specific standard, we next investigated whether observed enhanced liberation of a T-cell epitope by i20S proteasomes is due to an i20S proteasome-dependent higher substrate degradation rate or the result of altered cleavage site usage. To test this, the LLO-derived 27-mer polypeptide was digested with s20S liver pro-
Val-12, lacking correct C-terminal cleavage, versus the relative amount of the generated T-cell epitope and precursor. The data shown in Fig. 3C demonstrate that the more efficient generation of both the LLO epitope and the precursor peptides is due to significantly enhanced cleavage site usage of Leu-14 ↓ Lys-15 by i20S proteasomes compared with the cleavage at Val-12 ↓ Tyr-13. Remarkably, the usage of both analyzed N-terminal cleavage sites is also enhanced by i20S proteasomes, suggesting that the precursor, the antitope, and the epitope are generated by concerted dual cleavages (Fig. 3A). These data also demonstrate that frequently observed cleavages within an epitope do not necessarily have to result in a concomitant down-regulation of epitope production.

To control that the observed up-regulation is not a side effect of liver i20S proteasomes in infected mice, we also tested proteasomes from small intestines that constitutively contain high levels of i20S proteasomes that are not further increased after infection (16) and 20S proteasomes isolated from livers of infected IFN−/−-deficient mice that contain mainly standard proteasomes. As shown in Fig. 3D, the elevated epitope/antitope and precursor/antitope ratios in digest with small intestine proteasomes is not changed during the infection and no elevation of the epitope/antitope and precursor/antitope ratio was observed in IFN−/−-deficient mice that produce only negligible amounts of i20S proteasomes (supplemental Fig. S1).

To further assess the strength of the antitope approach, we extended our study to three other substrates and generated epitopes of different origin. Previous data had indicated that the generation of the murine Hsp60 epitope is not affected by i20S proteasomes. Digestion of a murine Hsp60-derived 30-mer polypeptide containing the epitope resulted in the generation of the correct T-cell epitope (Lys-10-Asp-18), a precursor peptide (Ser-5-Asp-18), and an antitope (Ser-5-Ile-16), the latter being the result of cleavage between Ile-16 ↓ Ser-17 (Fig. 4A). As observed for the LLO substrate, degradation of the Hsp60 30-mer polypeptide is considerably accelerated in the presence of i20S proteasomes (Fig. 4B). Concomitant with the enhanced degradation rate, the production of the epitope (Lys-10-Asp-18), the precursor peptide (Ser-5-Asp-18), and the antitope (Ser-5-Ile-16) is also enhanced by i20S proteasomes from Listeria-infected mice or from small intestine (Fig. 4C). However, standardization of the Hsp60 epitope and precursor peptides against the Hsp60 antitope reveals that there is no change in the preference of cleavage site usage by i20S proteasomes (Fig. 4D). Thus, in contrast to the LLO epitope, increased Hsp60 epitope generation is closely connected with accelerated substrate turnover.

In the last set of experiments we studied the generation of two predicted viral MHC class I epitopes derived from proteins of the Coxsackie virus B3 (CVB3) (Fig. 5A). Again, i20S proteasomes reveal an accelerated turnover of both the CVB3 P2C- and CVB3 P3D-derived polypeptide substrates (Fig. 5B). Despite the fact that the differences in epitope precursor and antitope production by i20S proteasomes or s20S proteasomes are not striking (Fig. 5C), standardization with the CVB3 P2C antitope Gln-13-Tyr-22 demonstrates that immunoproteasomes exhibit a clear preference for the usage of the correct C-terminal Phe-23 ↓ Ala-24 cleavage site compared with
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**DISCUSSION**

Previous in vitro analyses have shown that the presence of immunosubunits can alter the preference of proteasomal cleavage site usage in a given substrate, implying this to be the major reason for the frequently observed increase of i20S proteasome-dependent antigen presentation. In fact, in most cases studied (both in cells and in vitro) this conclusion seemed to be supported by the observed increase in i20S proteasome-dependent specific MHC class I antigen presentation. However, the finding that substrate degradation in vivo is in many cases accelerated in the presence of i20S proteasomes also raised the possibility that a general increase in peptide quantity due to enhanced substrate turnover may also serve as valid explanation for improved antigen presentation.

Thus, only if there exists a clear yes or no answer for the i20S proteasome dependence of an epitope, as has been shown for the hepatitis B virus core antigen and others, is a conclusion concerning cleavage site preferences straightforward (10, 13, 24, 25). Regarding the hepatitis B virus core antigen it became for the first time evident that incorporation of immunosubunits induces structural changes in the 20S proteasome core and that alterations in cleavage site preference do not necessarily require a novel catalytic site (10).

However, in most cases it remained difficult to distinguish whether improved CD8⁺ T-cell epitope presentation is due to increased substrate turnover or to specifically enhanced generation of the epitope itself. Part of the problem is inherent to mass spectrometry, which does not allow differential quantification of proteasomal peptide products.

Studying the generation of the *Listeria* LLO epitope we were faced with exactly this problem. In vivo experiments showed that i20S proteasomes enhanced the presentation of the LLO(297–304) (Ala-7-Leu-14) epitope in target cells; in agreement with this, in vitro digests revealed an apparently preferential generation of the LLO epitope or its precursor Ser-4-Leu-14 (LLO(297–304)) peptides. The fact that i20S proteasomes degraded the LLO substrate much faster than standard proteasomes made it, however, difficult to discern whether improved epitope generation is due to altered C-terminal cleavage site preference or to accelerated turnover of the LLO substrate.

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**FIGURE 4. Murine Hsp60 epitope generation is independent of i20S proteasomes.** A, scheme of the substrate peptide derived from Hsp60(171–200) used for in vitro digests is shown. The epitope Lys-10-Asp-18 and precursor Ser-5-Asp-18 generated by proteasomes are indicated as black bars and the related antitope Ser-5-Ile-16 as a white bar. B, substrate degradation by s20S proteasomes (WT liver proteasome) (open squares) and immunoproteasomes (infected liver at day 2 p.i.) (filled squares) was analyzed by HPLC-ESI-MS and is shown as means (± S.E.) of four independent experiments. C, the epitope, precursor, and the antitope fragments generated by the proteasomes from *Listeria*-infected liver within a 2-h digest were analyzed by HPLC-ESI-MS. The fragment amounts obtained in a digest with i20S proteasomes isolated from small intestines (s.i.) are shown at the right side of each panel (striated bars). The means (± S.E.) of four independent digests are shown. Statistical significance regarding the control was calculated by one-tailed heteroscedastic t test (*, p < 0.05). D, the fragments to antitope ratios show no i20S proteasome dependence for the generation of the Hsp60 epitope (squares) and precursor (diamonds); antitope (dotted gray line and circles) was set as 1. Open symbols represent s20S proteasomes, filled symbols the immunoproteasomes. Means of the quotients obtained from data of four independent experiments are shown.
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We therefore analyzed the generation of a substrate-specific, but epitope-independent, peptide that is cleaved by both standard and immunoproteasomes and thus compete with the generation of a functional epitope. As such, we first defined the Ser-4-Val-12 (LLO(294–302)) peptide as an antitope whose generation is due a cleavage within the epitope.

Interestingly, i20S proteasomes from T2.27 cells or from the liver of infected mice also generated the antitope with increased efficiency. Similar observations from other antigens, i.e. cleavage within the epitope sequence, were previously interpreted as being a sign for preferential epitope destruction by immunoproteasomes. However, when the amount of LLO epitope and precursor was normalized in correlation to the amount of antitope generated by both s20S and i20S proteasomes, it became evident that the correct C-terminal cleavage was enhanced in the presence of i20S proteasomes by a factor of approximately three in comparison to the antitope. Thus, the increased production of the LLO(294–304) T-cell epitope by i20S proteasomes was indeed the consequence of altered C-terminal cleavage site usage and not a consequence of accelerated substrate turnover. Our experiments also show that utilization of an antitope peptide for the discrimination of proteasomal cleavage site preferences is not restricted to the LLO substrate but can be extended to other epitopes, as demonstrated here for the two CVB3-derived epitopes, i.e. P2C and P3D.

For P3D, two antitope peptides could be selected for cleavage site analysis. One of the antitope peptides shared the N-terminal residue and the other peptide the C-terminal residue with epitope sequence. In consequence, the partial overlap of the antitope with the C and N termini of the epitope allowed the determination of the cleavage site preference for either the C-terminal or N-terminal cleavage required for epitope liberation.

An important and often neglected issue is that enhanced C-terminal cleavage alone as usually assigned to i20S proteasomes does not sufficiently explain the improved epitope liberation, because this also requires a concomitant N-terminal cleavage. As such, the widespread opinion that immunoproteasomes support epitope generation due to their capacity to cleave more efficiently behind hydrophobic, i.e. potential anchor residues, falls short of the real requirements for antigen processing. The analysis of the LLO substrate revealed that i20S proteasomes also enhance N-terminal cleavage. Increased and coordinated dual cleavage events were previously shown for the function of PA28/20S proteasome complexes, whereby PA28 seems to induce subtle structural changes on the 20S core proteasomes without affecting the active site themselves (26, 27). In addition, induction of structural changes by the β5i subunit was shown to result in altered cleavage site preferences of the 20S proteasome core complex. It is unclear how far double cleavage events by i20S proteasomes and those induced by PA28 are mechanistically related, but it is interesting to note that apparently both the IFN-induced i20S proteasomes and PA28 exert structural changes onto the 20S core that allow for more efficient double cleavage events and concomitant enhanced epitope production.

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