The Cleavage Preference of the Proteasome Governs the Yield of Antigenic Peptides

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Summary

Proteasomes degrade endogenous proteins in the cytosol. The potential contribution of the proteasome to the effect of flanking sequences on the presentation of an antigenic epitope presented by the major histocompatibility complex class I allele L^d was studied. Peptides generated in cells from minigenes coding for peptides of 17– and 19–amino acid length were compared with the in vitro 20S proteasome degradation products of the respective synthetic peptides. The quality of generated peptides was independent of ubiquitination. In vivo and in vitro processing products were indistinguishable with respect to peptide size and abundance. Altering the neighboring sequence substantially improved the yield of the final antigenic nonapeptide by 20S proteasome cleavage. These results suggest that, in addition to the presence of major histocompatibility complex class I allelic motifs, the cleavage preference of the proteasome can define the antigenic potential of a protein.

In the MHC class I pathway of antigen presentation, proteins are degraded in the cytosol, and resulting peptides are transported into the endoplasmatic reticulum, where they assemble with the MHC class I heavy chain and β_2-microglobulin to the trimolecular MHC complex. This complex is subsequently exported to the plasma membrane to be recognized by peptide-specific CTL. Insertion of the sequence coding for the antigenic peptide into unrelated proteins does not prevent processing and presentation (1). However, the insertion of the sequence of the murine CMV (MCMV)[1]–derived ^{168}YPHFMPTNL^{176} peptide (2) into the hepatitis B virus e-protein (HBe) revealed a strong positional effect in antigen processing, which could be rescued by flanking the epitope with multiple alanines (3). This modulatory effect of flanking sequences has also been seen using constructs expressing peptides of influenza proteins (4, 5).

There is evidence that the proteasome is involved in antigen processing. Two subunits of the proteasome, LMP2 and LMP7, are encoded in the MHC (6–9), and mice lacking LMP7 (10) are compromised with respect to MHC class I molecule expression and antigen expression (11). Inhibitors of the proteasome block the generation of peptides presented by MHC class I molecules (12). Expression of a protein fused to ubiquitin improved antigen presentation (13). Furthermore, processing of a complex protein required ubiquitination (14) for degradation by the proteasome in vivo. The effect of flanking residues on the presentation of YPHFMPTNL may be associated with ubiquitination and the proteasome degradation pathway. We find that the positional effect of flanking amino acids operates in absence of ubiquitination. The comparison of the peptides generated in vivo and in vitro shows that the positional effect on antigen presentation can be completely explained by the function of the 20S proteasome.

Materials and Methods

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses were generated according to Del Val et al. (3). The minigenes were generated by PCR of the corresponding chimeric HBe gene starting with an initial methionine. The recombinant minigenes were coding for a 17-mer (MDIGYPHFMPTNLGDPY) or a 19-mer (MDIGAYPHFMPTNLAGDPY). The fidelity of genetic engineering procedures was confirmed by DNA sequence analysis of plasmid inserts. Genes were inserted into the TK locus of vaccinia virus by homologous recombination.

Cytolytic Assay of Naturally Processed Peptides. BALB.SV cells were infected with recombinants at a multiplicity of infection of 4 PFU per cell for 7 h. Infected cells served as targets in a standard ^{51}Cr release assay. The specific CTL generated by in vitro restimulation with 10^{-8} M nonapeptide were added in graded numbers (3). Data represent the mean percentage from six replicate cultures.

Isolation of Naturally Processed Peptides. Naturally processed peptides were extracted from cells as described by Rötzschke et al. (15). Briefly, TFA-soluble material was separated first on a Sephadex G25 column and subsequently on an HPLC reversed-

[1]*Abbreviations used in this paper:* HBe, hepatitis B virus e-protein; LLnL, *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal; LMP, low molecular weight protein; MCMV, murine CMV; MG-132, *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal.

phase column (Superpac pepS; Pharmacia LKB, Freiburg, Germany). Eluent A was 0.1% TFA; eluent B was 70% acetonitrile containing 0.1% TFA. The gradient was 25–90% eluent B in 14 min, the flow rate was 0.8 ml/min^{-1}, and fraction size was 0.8 ml. Fractions were resuspended and diluted in RPMI 1640 medium. ^{51}Cr-labeled P815 cells were added and, after 1 h of incubation, probed by CTL. Fractions were tested in triplicate. The nonapeptide elutes in fraction 15 and the noncleaved minigene products elute in fraction 19.

Assay of Proteolytic Activity. 20 μg of the synthetic polypeptides (MDIGYPHFMPTNLGDPY; MDIGAYPHFMPTNLAGDPY) and 1 μg of 20S proteasome from BALB.SV cells prepared according to Boes et al. (16) were incubated at 37°C for 24 h in a total volume of 300 μl assay buffer (20 mM Hepes/KOH, pH 7.8, 2 mM $MgAc_2$, 1 mM dithiothreitol). After titration of the digestion mixture in 10-fold dilution steps (10^{-6}–10^{-12} M) the specific lysis was determined by ^{51}Cr release assay. Incubation was performed either with 5% FCS in RPMI 1640 or under serum-free conditions using 5% BSA in RPMI 1640. The increase in antigenicity was calculated from the half-maximal lysis of the linear range of the regression curve. The nonapeptide served as a standard.

Mass Spectrometric Analysis. 10 μl of proteasome digest was separated by reversed-phase HPLC (SMART-System equipped with a μRPC C2/C18 SC 2.1/10 column; Pharmacia LKB) (eluent A, 0.1% TFA; eluent B, 70% acetonitrile containing 0.09% TFA; gradient, 20–95% eluent B in 15 min, flow rate 50 μl/min^{-1}) and analyzed on-line by a tandem quadrupole mass spectrometer (model TSQ 7000; Finnigan MAT, Bremen, Germany) equipped with an electrospray ion source. Each scan was acquired over the range mass/charge (m/z) 300–1,300 in 2 s. The sequences of the major cleavage products were determined by fragmentation of peptides with argon atoms in tandem mass experiments. Less-abundant peptides were identified by calculation of the molecular mass from the m/z peaks of the double-charged ions and coelution with synthetic peptides of potential cleavage products. The amount of peptides was determined by the intensity of the ion current of the double-charged ions using the synthetic peptides as a standard.

Proteasome Inhibition Assay. Inhibitor studies were done with 1 μg of proteasome using 20 μg of the 19-mer peptide in 300 μl of assay buffer as described above, adding 5–50 μM of the aldehyde inhibitors *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL) (Sigma Chemical Co., St. Louis, MO) or *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal (MG-132, similar to MG-115 [12]) (Myogenics, Cambridge, MA). After 24 h at 37°C, the samples were subjected to mass spectrometry analysis. The percentage of inhibition was calculated from the intensity of the main degradation products (YPHFMPTNL and MDIGAYPHFMPTNL).

HPLC Separation of Processed Peptides. 50 μl of the digest was separated by reversed-phase HPLC as described in mass spectrometric analysis but using another gradient (5–50% B in 30 min, 50–95% B in 10 min, flow rate 100 μl/min^{-1}). 100-μl fractions were collected. For the quantification of the antigenicity of the fractions, 10-fold dilutions were tested in triplicate in a 3-h ^{51}Cr release assay. The antigenic peptides were identified by mass spectrometry and by coelution of synthetic peptides. The activity of the 10^{-2} dilution is shown in Fig. 5.

Results and Discussion

To compare antigen processing in cells with peptide degradation in vitro by the 20S proteasome, two minigenes coding for short peptides were constructed. One minigene codes for a 19-mer peptide representing the antigenic MCMV epitope, a biterminal alanine spacer, and the local flanking amino acids of HBe (^{1}MDIGAYYPHFMPTNLAGDPY^{19}). The other minigene encodes a 17-mer peptide (^{1}MDIGYYPHFMPTNLGDPY^{17}) without the alanines. The minigenes were expressed in cells by recombinant vaccinia viruses. As a first step, antigen presentation from minigene products was compared with complete HBe proteins. As observed before (3), alanine spacing at both termini of the naturally processed nonamer sequence, YPHFMPTNL, improved antigen presentation. Extending previous data using multiple alanines, a single biterminal alanine was also sufficient (Fig. 1 *A*). Thus, the minigenes also showed a positive alanine effect on antigen presentation. There was no detectable quantitative difference with respect to antigen presentation between minigenes and the HBe constructs. Whereas recombinant HBe should be susceptible to ubiquitin-dependent degradation, minigenes expressing short peptides that lack lysine residues important for ubiquitination (17) should not. Therefore, the positional effect on antigen presentation operates independently of ubiquitination of the antigenic polypeptide.

Total extracts from cells infected with the vaccinia recombinants were separated by reversed-phase HPLC to associate the biological activity with defined peptides. P815 target cells were incubated with individual HPLC fractions to allow peptides to associate with L^d molecules and then exposed to nonapeptide-specific CTL. These CTL detect not only the MCMV nonapeptide but also related peptides (2). Only the results for the extracts from cells expressing the minigenes are shown, because the data with the chimeric

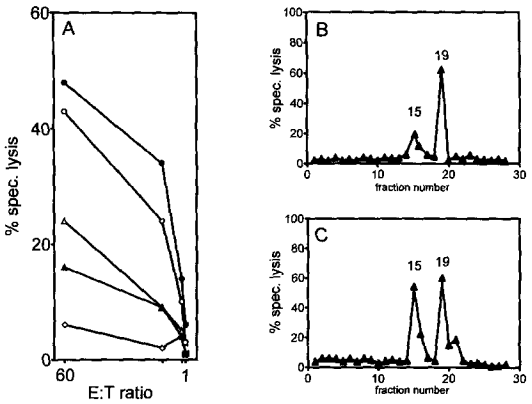

Figure 1. Rescue of antigen processing and presentation by alteration of sequences flanking the epitope. (*A*) The nonapeptide sequence ^{168}YPHFMPTNL^{176} of the MCMV IE1 protein, which is naturally presented by the MHC class I allele L^d (2), with (*open circles*) and without (*open triangles*) flanking alanines was inserted close to the NH_2 terminus of the HBe protein. Similarly, minigenes coding for parts of this sequence (MDIGAYPHFMPTNLAGDPY [*filled circles*] and MDIGYPHFMPTNLGDPY [*filled triangles*]) were constructed and tested for antigen presentation by specific CTL. HBe-vac (*open diamonds*) served as negative control. (*B* and *C*) Detection by CTL of naturally processed antigenic peptides extracted from cells infected with recombinant minigenes expressing the 17-mer MDIGYPHFMPTNLGDPY (*B*) or the 19-mer MDIGAYPHFMPTNLAGDPY (*C*).

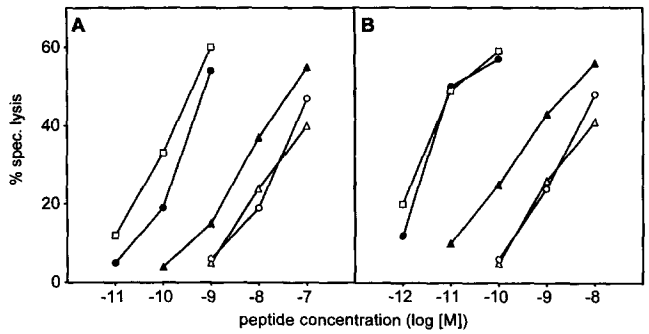

Figure 2. Increase in antigenicity after degradation of synthetic peptides by the 20S proteasome. The synthetic peptides MDIGAYPHFMPTNLAGDPY (*circles*) and MDIGYPHFMPTNLGDPY (*triangles*) were incubated with 20S proteasome. The antigenicity of digestion products (*filled symbols*), undigested peptides (*open symbols*), and the nonapeptide YPHFMPTNL (*open squares*) were quantified by ^{51}Cr release assay under serum-containing (*A*) and serum-free (*B*) conditions.

HBe proteins after biterminal flanking with one alanine were essentially identical to previously reported data (3). All constructs generated biological activity that coeluted with the synthetic nonapeptide. Dilution of active fractions (data not shown) showed that the peak generated from alanine-flanked constructs reproducibly yielded ~25-fold more activity (Fig. 1, *B* and *C*). Furthermore, additional peaks of activity coeluted at a position of full-length 17-mer and 19-mer peptides. These additional peaks were not seen with the full-length HBe constructs.

The effect of alanine spacing on peptide presentation could either reflect differences in degradation, transport, or a combination of both effects. Degradation of polypeptides by 20S proteasome in vitro has been studied in previous reports (16, 18, 19). To test whether sequence differences can alter the proteasome cleavage preference in vitro, the two synthetic peptides of 17-mer and 19-mer substrates were analyzed for biological activity after proteasome digestion (Fig. 2).

It has been observed before (20) that some longer peptides, in the reported example a 16-mer peptide, can be bound to MHC class I molecules for CTL recognition, but that the natural ligand is recognized 1,000-fold better. Accordingly, the undigested 17-mer and 19-mer substrates had little activity, and at least 10^{-8} M of peptide was required for target cell formation, whereas the natural processed

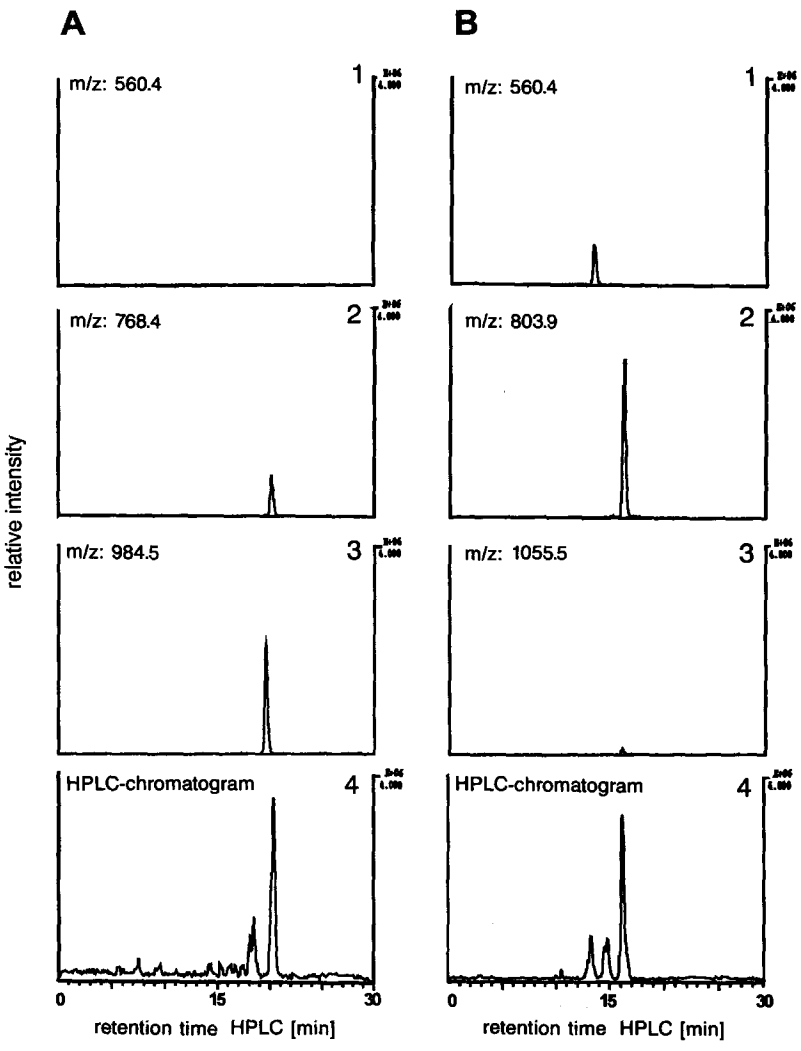

Figure 3. Characterization of proteasome digestion products by mass spectrometry. 20S proteasome-degraded peptides of the 17-mer (*A*) and the 19-mer (*B*) were separated by reversed-phase HPLC and analyzed on-line by mass spectrometry for identification of individual peptides in complex mixtures of distinct peptides (22). The separation is documented by the HPLC chromatogram (4) and by the ion current of twofold positive-charged peptide ions detected by mass spectrometry (1–3). *A1* and *B1* (m/z 560.5) show the mass peak of the nonapeptide YPHFMPTNL, *A2* (m/z 768.4) the 13-mer MDIGYPHFMPTNL, and *B2* (m/z 803.9) the 14-mer MDIGAYPHFMPTNL. The remaining uncleaved substrates are shown in *A3* (m/z 984.5) MDIGYPHFMPTNLGDPY and *B3* (m/z 1,055.5) MDIGAYPHFMPTNLAGDPY.

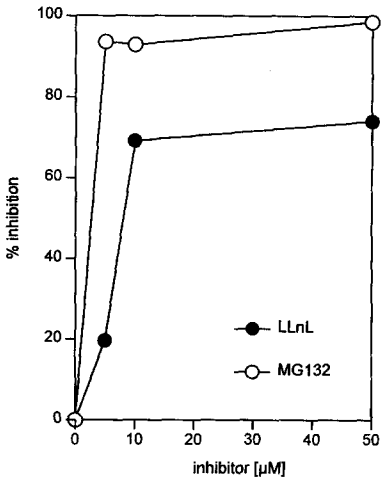

Figure 4. Effect of peptide aldehydes on the peptide-degrading activity of 20S proteasomes. The synthetic 19-mer (MDIGAYPHFMPTNLAGDPY) was subjected to 20S proteasome digestion in the presence of increasing concentrations of inhibitor. The reduced yield of the main cleavage products YPHFMPTNL and MDIGAYPHFNPTNL is expressed as the percentage of inhibition.

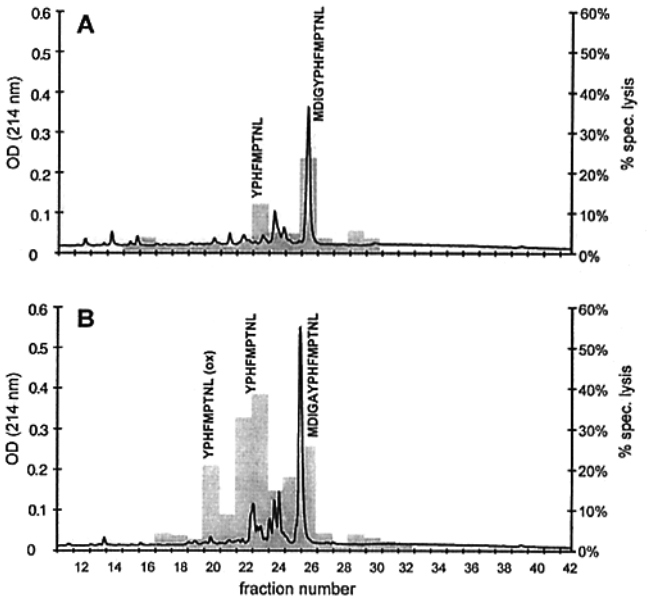

Figure 5. CTL recognition of 20S proteasome–degraded peptides. The yield and the identity of antigenic peptides processed by the 20S proteasome from the 17-mer (*A*) and 19-mer (*B*) were probed by nonapeptide-specific CTL. The OD curve and the specific lysis (*gray columns*) are shown for individual HPLC fractions.

nonamer is reactive at a concentration between 10^{-11} and 10^{-12} M. 20S proteasome incubation of the 17-mer peptide led only to a fourfold activity increase (Fig. 2 *A*). However, the effect on the alanine-flanked peptide was striking, and the activity increased 160-fold.

Sherman et al. (21) reported that under certain conditions a serum protease, angiotensin-converting enzyme, can contribute to the activation of an inactive peptide by removing a COOH-terminal dipeptide. To test the potential proteolytic degradation of proteasome products by serum components, the external loading of the peptides and the detection of the biological activity was carried out under serum-free conditions (Fig. 2 *B*). There were no differences between presence or absence of serum. We conclude that the processing of the external loaded peptides was entirely due to the function of the proteasome.

To characterize major degradation products and to identify individual peptides in the digests, the materials were separated by HPLC and analyzed on-line by mass spectrometry (22) (Fig. 3). Only 31% of the 17-mer substrate was degraded. This was not due to substrate length or proteasome activity. The 13-mer MDIGYPHFMPTNL represented the main cleavage product. At the position of the nonapeptide YPHFMPTNL, no signal was detectable. In contrast, the 19-mer peptide was almost completely digested (98%). The 14-mer MDIGAYPHFMPTNL was identified as the major product (63%). The antigenic nonapeptide constituted 17% of the products and explained the strong increase in biological activity.

To consider the potential contribution of contaminating proteases, the 20S proteasome degradation of the 19-mer was carried out in the presence of the peptide aldehyde 20S proteasome inhibitors LLnL and MG-132, described by Rock et al. (12) (Fig. 4). The mass spectrometry analysis of the major degradation products YPHFMPTNL and MDIGYPHFMPTNL revealed a strong inhibition, almost 100% of the 20S proteasome by the potent inhibitor MG-132. As already observed by Rock et al., the inhibition of the proteasome by LLnL was weaker. These data emphasize that the observed processing of the peptide substrates represents a 20S proteasome function.

To identify the peptides responsible for the biological activity, the antigenicity of degradation products was analyzed after reversed-phase HPLC separation. In the 17-mer digest, the CTL detected an activity in fraction 26 (Fig. 5 *A*), where both substrate and 13-mer (MDIGYPHFMPTNL) coelute. Therefore, the additional peak detected in cell extracts from minigenes (Fig. 1, *B* and *C*) may also contain the full-length peptide and related cleavage products. Both peptides have low intrinsic antigenicity and are only recognized because of their large quantity.

The activity in fraction 23 comigrates with the antigenic nonapeptide YPHFMPTNL. Thus, only a minor fraction of the 17-mer peptide is processed to the nonapeptide. Analysis of the 19-mer digest (Fig. 5, *B*) identified the major product MDIGAYPHFMPTNL in fraction 26. The bulk of the biological activity resides in fractions 22 and 23, where YPHFMPTNL and AYPHFMPTNL coelute. According to mass spectrometry, the 10-mer constitutes only 5% of the products (not shown). The activity in fraction 20 coelutes with a peptide whose molecular mass is 16 mass units higher than the nonapeptide. The assumption that the methionine of this peptide is oxidized was confirmed by coelution with the oxidized synthetic peptide, YPHFMPTNL(ox). Thus, the sequence alterations affecting antigen presentation in vivo are truly reflected by the in vitro

20S proteasome degradation profiles and the generation of a naturally processed nonamer.

In vitro, the 20S proteasome degrades polypeptides progressively (18). Resulting peptides have a size range appropriate for MHC class I binding (23), and antigenic peptides that are naturally presented by MHC molecules are among the degradation products (17, 19). Induction of the MHC-linked LMP products alters the composition and cleavage specificity of the proteasome (17, 24, 25). The 20S proteasome constitutes the proteolytic core of the 26S complex, which is essential in the ATP-ubiquitin–dependent degradation pathway (26, 27). The claimed ubiquitin dependence of MHC class I–restricted processing of proteins (14) questions the validity of results obtained with the 20S proteasome in vitro. Reinvestigation of the same model system, however, did not confirm the ubiquitin requirement (28). We describe here that the same sequence alteration in an endogenously processed protein, in an endogenously processed peptide, and in a synthetic peptide all result in the same relative abundance of the antigenic nonapeptide. This provides a new line of evidence that the proteasome is involved in antigen processing in the MHC class I pathway. The observed cleavage preference of the proteasome operates independently of ubiquitination.

In the MCMV model studied by us, the observed peptide presentation differences are decisive for the protective capacity of a vaccination protocol (3). If the proteasome codefines the molecular basis of antigenicity, then proteasome specificity is a key question. Short fluorogenic peptides have been used to describe several peptide-hydrolyzing activities (29, 30). This characterization has been questioned because most peptide bonds of polypeptide substrates are cleaved (25) and because cleavage is affected by peptide size (23). Degradation kinetics established leucine as the first and major cleavage site in our example. The alanine in position 15 of the 19-mer controls the degree of the primary cleavage behind the leucine, and the alanine in position 5 enhances the further degradation of the 14-mer intermediate. The fact that alanine insertions modulate the proteasome cleavage preference suggests that interactions between substrate and enzyme extend over a larger area. Concepts for a coordinated activity between distant active sites of the enzyme complex have been proposed (23, 24). The insertion of residues into a substrate could improve the coordinated activity of proteolytic centers of the protease due to spacing of two scissile bonds. Alternatively, alterations of peptide conformation by exchange or insertion of residues could improve substrate binding to the active sites of the proteasome. Thus, local residues in the primary sequence and in intermediate peptides can be decisive for the relative abundance of final products.

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