Antigen Processing of a Short Viral Antigen by Proteasomes*

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Mass spectrometry (MS)-based methods coupled to reverse phase chromatography separation are a useful technology to analyze complex peptide pools that are comprised of different peptides with unrelated sequences. In antigen presentation, proteasomes generate a set of short peptides that are closely related and overlapping and in some instances may even have identical retention times and identical masses. In these situations, micro-liquid chromatography-MS/MS focused on each theoretical parent ion followed by manual interpretation optimizes the identification of generated peptides. The results suggest that the degradation of short antigens by the proteasome occurs by sequential cleavage.

Recognition of virus-infected cells by cytotoxic T lymphocytes requires prior proteolytic processing of viral proteins (1). This degradation generates short peptides that are translocated to the endoplasmic reticulum lumen by transporters associated with antigen processing, assembled with major histocompatibility complex class I heavy chain and B2-microglobulin, and transported to the cell membrane (1). In infected cells, processing predominantly takes place in the cytosol by the combined action of proteasomes and degradative peptidases. Processing generates a broad diversity of peptides including a small fraction of the correct epitope or N-terminally extended precursors (1) that are utilized for major histocompatibility complex class I antigen presentation. These epitope precursor peptides require N-terminal trimming by aminopeptidases either in the cytosol or in the endoplasmic reticulum (2). We are interested in the identification of the set of processed peptides generated from short viral proteins by antigen processing by the proteasome and/or other proteases.

In a previous work (3), we studied antigen processing from recombinant vaccinia viruses encoding two closely related minigene products encompassing murine cytomegalovirus pp69 immunodominant nonamer epitope (4). By using protease inhibitors, we defined a role for proteasomes in physiological intracellular antigen processing of short antigens. One of the minigenes (m19) codes for a 19-mer peptide, MDIGAYPHFMPTNLAGDPY. It represents the immunodominant murine cytomegalovirus 9pp89 epitope (YPHFMFPTNL) and the local flanking amino acids of HBe carrier protein. In addition, it presents a biterminal alanine spacer between the 9pp89 core and HBe flanking residues, which was previously reported to enhance antigenicity (5). In this classic paper Eggers et al. (5) characterized the proteasome digestion products of this m19 peptide. The authors identified two cleavage products; the 1–14 peptide (MDIGAYPHFMPTNL) as the major degradation product and also the 9pp89 epitope (YPHFMFPTNL14) were detected. As the proteasome is a multicatalytic protease with three different activities (6), some authors have demonstrated a broad spectrum of processed peptides generated by the action of this protease on different proteins (reviewed in Ref. 7).

Accordingly, we can expect higher diversity in m19 cleavage experiments that are revealed with the classic MS2 analysis (5). In the last years immunoproteomics or mass spectrometry-based methods to study the targets of the immune response have been developed (8). With the standard automated micro-LC-MS/MS scan mode we detected four m19-derived cleavage products. Therefore we modified this technical approach involving micro-LC-MS/MS focused on each theoretical parental cleavage product followed by manual interpretation, and thus we optimize the detection of generated peptides. This strategy allow the identification of nine cleavage products. With these data, we suggest a sequential cleavage model for proteasome activity on the m19 peptide.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—The peptides were synthesized in an Applied Biosystems peptide synthesizer model 433A (Foster City, CA), purified, and found homogeneous by HPLC analysis.

HPLC Analysis of Peptides—Five nanomoles of each synthetic peptide was analyzed by reverse phase HPLC (SMART system equipped with a μRPC Sephasil C18 SC 2.1/10 column (GE Healthcare)). The eluents used were: A, 19.6% acetonitrile containing 0.1% trifluoroacetic acid; B, 70% acetonitrile containing 0.1% trifluoroacetic acid. The gradient was: 0–30.2% B in 18 min and 31.2–100% in 4 min, and the flow rate was 210 μl/min-1. Several runs were performed with each synthetic peptide, and their location inside the acetonitrile gradient was reproducible with a maximum error of one fraction (52 μl).

Proteasome Assays—Isolation and purification of 20 S proteasomes from rabbit psoas muscle used were performed

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2 The abbreviations used are: MS, mass spectrometry; LC, liquid chromatography; ESI, electrospray ionization; IT, ion trap; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
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FIGURE 1. Analysis by MALDI-MS of m19 synthetic peptide digested with purified proteasomes. In addition to m19 substrate, MALDI-TOF analysis detected three different molecular species numbered 1, 2, and 3. In all cases the sodium adduct and the peptide with an oxidized Met in addition to the sodium adduct were detected and marked as single asterisk and double asterisk, respectively. Species number 1 could correspond to either 6–15 or 5–14 peptides. The m/z number 2 could correspond to either 6–16 or 4–14 peptides. Finally, four different peptides (1–14, 2–16, 3–17, and 4–18) could have the m/z number 3.

RESULTS AND DISCUSSION

As a first step, proteasomes were isolated and purified (9). The reaction conditions for proteasome were as published (10).

Mass Spectrometry Analysis—MALDI-TOF mass spectrometry was performed in a Reflex IV instrument (Brucker-Franzen Analytik, Bremen, Germany) operating in the positive ion reflection mode. Twenty-five microliters of sample were dried, resuspended in 1 μl of 0.1% trifluoroacetic acid in a 2:1 solution of water:acetonitrile, and mixed with 1 μl of saturated α-cyano-hydroxyhydrocinnamic acid matrix in the same solution. One microliter of the mixture was dried and subjected to analysis.

Aliquots of total digestions were dried and dissolved in 10 μl of 0.5% acetic acid in water and sequenced by quadrupole ion trap micro-HPLC (Biobasic C18 column, 150 × 0.18 mm (Thermo Electron, San Jose, CA) electrospray MS/MS in a Deca XP LCQ mass spectrometer (Thermo Electron). The eluents used were: A, 0.5% acetic acid in water; B, 80% acetonitrile containing 0.5% acetic acid. The gradient was 0 – 40% B in 24 min and 40–100% in 5 min, and the flow rate was 1.5 μl/min. We used the MS/MS mode focused to each hypothetical parental peptide with a isolation width (m/z) of 1.5 Da. The charge and the mass of the ion species were determined by high resolution sampling of the mass/charge rank. Collision energy and ion precursor resolution were improved to optimize the fragmentation spectrum.

As a first step, proteasomes were isolated and purified (9). Next, m19 synthetic peptide was incubated with purified proteasomes. After that, the cleavage products generated by this protease involved in m19 processing were analyzed by mass spectrometry. In a first approximation MALDI-MS analysis of total digestion was carried out. This system allowed the detection of three different peptidic species derived from the m19 sequence (Fig. 1). Species number 3 had identical m/z with the 1–14 peptide detected as the major cleavage product by Eggers et al. (5). To elucidate the nature of the two additional products 6–14 peptides were identified as cleavage products. No peptides were detected that arose from cleavages within the 9pp89 core in both MALDI-TOF and ESI-IT analysis.

Usually the automated MS/MS scan mode allows for the identification of predominant parental peptidic species, but we are interested in the identification of the largest spectrum of products generated by the proteasome activity. The potential digestion products of m19 are closely related and the collection is highly complex. Indeed, in the mixture of digestion products of m19 peptide by the proteasome 35 theoretical different peptides are possible that contain the cytomegalovirus 6–14 nonamer core, that is that are immunologically relevant. As the m19 sequence presents two Met (easily oxidized) located at the N terminus and at residue number 10, all processed relevant peptides could appear also as the oxidized molecular ion. In addition, five of these peptides present the two Met residues in their sequence. In summary, we need to detect 42 distinct molecular ions that correspond to 80 relevant molecular species derived from the m19 sequence; some of them have the same hydrophobic properties and some have identical daughter ions produced by MS fragmentation.

As the hypothetical cleavage products are overlapping peptides because they have an identical nonamer core and present staged length, their hydrophobic properties in reverse phase HPLC could be related. In addition, the two residues flanking the 9pp89 epitope are symmetrical, and thus, their corresponding peptides (A9pp89 and 9pp89A or GA9pp89 and 9pp89GA) present identical HPLC retention properties as well as m/z values. Previous HPLC separation analysis (data not shown) indicated a short separation of a maximum of 4 to 5 fractions between the two extreme peptides, m19 and 9pp89 epitope (“YPFMPTNL”14). This is true even when the gradient used for elution of the synthetic peptides from the reverse phase HPLC column was rather
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flat (see “Experimental Procedures”). Thus, several overlapping peptides could co-elute in the same HPLC fraction. A general limitation of the analysis of complex mixtures by MS is the inability to discriminate isomers because MS/MS data are often ambiguous when multiple components are fragmented simultaneously. This might explain the limited improvement obtained with HPLC-ESI-IT over MALDI-MS analysis. Thus, we studied each one of the 35 theoretical different peptides that contain the cytomegalovirus 6–14 nonamer core in successive micro-HPLC assays to explore all possible products of the proteasome activity. Because only ions of selected mass-to-charge ratio are monitored, their scan mode generally provides higher sensitivity than does the full scan mode. Thus, by using micro-LC-MS/MS with fixed precursor to analyze each theoretical doubly charged ion derived from m19 sequence and performing a continuous operation in MS/MS under standard fragmentation conditions, we can acquire more information on the studied biological process.

Fig. 3 shows the interpretation of overlapping products derived from the m19 substrate. Five different molecular ions could be detected by MS/MS fragmentation of the doubly charged ion at $m/z$ 810.9. This is the most complex indecisiveness that exists among all potential overlapping peptides derived from the m19 sequence. This $m/z$ corresponds to five different 14-mers: the 6–19 peptide, both 2–16 and 3–17 peptides with their respective oxidized Met, and the 1–14 peptide with either Met-1 or Met-10 oxidized. As shown in the inset of Fig. 3, the mass spectrometer detects a molecular ion with this $m/z$ range around minute 36 of the HPLC gradient compatible with the five molecular species cited above. The fragment spectrum obtained was manually interpreted, yielding two different sequences that satisfactorily matched the pattern of fragment ions. This analysis discards the presence of three of five candidates and as shown in Fig. 3 demonstrates the existence of both 1–14 peptides with either Met-1 or Met-10 oxidized.

Fig. 2 (bottom) summarizes the results obtained with the micro-HPLC-MS/MS operating with the parental ion fixed for all isomeric peptide mixtures. The MS/MS fragmentation spectra were manually interpreted to identify all co-eluting overlapping peptides including those present in lower relative amounts.

Some peptides such as 1–17 were found in all cleavage experiments including the negative control with only the m19 synthetic peptide without proteases and were not considered. These results agree with some reports (11) that indicate that Asp-Pro bonds of synthetic peptides can undergo spontaneous cleavage under acid conditions such as those used in solubilization and purification of peptides prior to ESI-IT analysis.

These data show a broad spectrum of processed peptides including the four previously defined (see above, Fig. 2) and demonstrate that the proteasome generates a collection of peptides where most peptides were N-extended and had the correct C-terminal Leu.
Altogether we suggest the sequential cleavage model depicted in Fig. 4 as the major pathway followed by the proteasome. First, the proteasome would cut the m19 peptide between residues Gly-16 and Asp-17. In a second step, the two C-terminal residues (Ala-15 and Gly-16) of this product would be removed. Next, N-terminal Met and Asp residues would be cleaved. Afterward, the N terminus of this 3–19 product would continue to be trimmed residue by residue. This model accounts for three-fourths of the total amount of detected peptides. It has been proposed that the substrate interacts with the large inner surface of the proteasome core at undefined non-catalytic sites (12). In addition, some authors suggest that the substrate can be covalently attached to one active site in a transition state complex and then be attacked in turn by other active sites (13). Therefore, as the 1–14 peptide is the major cleavage product of m19 degradation (Fig. 1) and we detect mainly N-terminal step by step trimming, this model suggests that Leu-14 is fixed whereas adjacent catalytic sites remove sequentially these N-terminal residues. These N-extended peptides could be trimmed by aminopeptidases (2). In addition to cleavages inside the 9pp89 epitope, the different prediction algorithms for proteasome activity predict the C-terminal cleavage but not the N-end trimming observed in our experiments.

Alternatively, in each m19 molecule the proteasome may simultaneously perform two cleavages, one with relaxed specificity at the N terminus and the other one strongly focused on the preferred cleavage site after Leu-14.

With three other additional products detected (4–15, 2–16, and 3–17 peptides) no obvious specificity patterns were found. The most simple explanation could be that some m19 molecules were cut with a relaxed specificity in the two cleavages that generates these individual peptides independently.

In summary, our results show how the identification of highly related peptidic species in a highly selective and specific manner is possible and allow its application to elucidate the mechanisms of antigen processing.

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REFERENCES

1. York, I. A., Goldberg, A. L., Mo, X. Y., and Rock, K. L. (1999) Immunol. Rev. 172, 49–66
2. Saveanu, L., Fruci, D., and van Endert, P. (2002) Mol. Immunol. 39, 203–215
3. López, D., and Del Val, M. (1997) J. Immunol. 159, 5769–5772
4. Del Val, M., Volkmer, H., Rothbard, J. B., Jonjic, S., Messerle, M., Schickendantz, I., Reddehase, M. J., and Koszinowski, U. H. (1988) J. Virol. 62, 3965–3972
5. Eggers, M., Boesfabian, B., Ruppert, T., Kloetzel, P. M., and Koszinowski, U. H. (1995) J. Exp. Med. 182, 1865–1870
6. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
7. Kloetzel, P. M. (2004) Biochim. Biophys. Acta 1695, 225–233
8. Purcell, A. W., and Gorman, J. J. (2004) Mol. Cell. Proteomics 3, 193–208
9. Kisselev, A. F., Akopian, T. N., Woo, K. M., and Goldberg, A. L. (1999) J. Biol. Chem. 274, 3363–3371
10. Kisselev, A. F., García-Calvo, M., Overkleeft, H. S., Peterson, E., Pennington, M. W., Ploegh, H. L., Thornberry, N. A., and Goldberg, A. L. (2003) J. Biol. Chem. 278, 35869–35877
11. Skribanek, Z., Mezo, G., Mak, M., and Hudecz, F. (2002) J. Pept. Sci. 8, 398–406
12. Kisselev, A. F., Kaganovich, D., and Goldberg, A. L. (2002) J. Biol. Chem. 277, 22260–22270
13. Lowe, J., Stock, D., Jap, B., Zwicky, P., Baumeister, W., and Huber, R. (1995) Science 268, 533–539

3 D. López, O. Calero, M. Jiménez, M. García-Calvo, and M. Del Val, unpublished data.