

# Concerted Antigen Processing of a Short Viral Antigen by Human Caspase-5 and -10\*

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The generation of peptides presented by MHC class I molecules requires the proteolytic activity of the proteasome and/or other peptidases. The processing of a short vaccinia virus-encoded antigen can take place by a proteasome-independent pathway involving initiator caspase-5 and -10, which generate antigenic peptides recognized by CD8<sup>+</sup> T lymphocytes. In the present study, comparing single *versus* double enzyme digestions by mass spectrometry analysis, both qualitative and quantitative differences in the products obtained were identified. These *in vitro* data suggest that each enzyme can use the degradation products of the other as substrate for new cleavages, indicating concerted endoproteolytic activity of caspase-5 and -10.

The proteasome is the primary and major proteolytic complex involved in the cytosolic generation of epitopes from newly synthesized pathogen proteins (1). This multicatalytic endoprotease regulates the degradation of cellular proteins and produces a broad diversity of peptides. The immune system has not evolved specialized proteolytic mechanisms for cytosolic antigen processing but utilizes this phylogenetically ancient catabolic pathway to mark pathogen-infected cells. Among the vast array of peptides generated by the proteasome, a small fraction constitutes the correct epitope or NH<sub>2</sub>-terminally extended precursors, both of which can be utilized for MHC class I antigen presentation. These epitope precursor peptides may be N-terminally trimmed by aminopeptidases in the cytosol (2) or after transport by TAP transporters to the lumen of the endoplasmic reticulum (3, 4). Lastly, binding to nascent MHC class I molecules generates the antigenic complex recognized at the infected cell surface by specific CD8<sup>+</sup> cytotoxic T lymphocytes.

In addition to proteasome-independent and TAP-independent antigen processing secretory pathways (reviewed in Refs. 5, 6), the endoproteolytic activity of tripeptidyl peptidase II was described as the first enzymatic activity able to generate pathogen-derived peptides in the cytosol when proteasomes are inhibited (7, 8). Endoproteolytic activities of caspases (9), nardilysin (10), and insulin-degrading enzyme (11) were impli-

cated recently in antigen processing. In the caspase study, an additional proteasome-independent pathway for antigen processing of the 19-amino acid-long gene product encompassing murine CMV immunodominant epitope was characterized (9). This pathway operates when apoptosis is induced by vaccinia virus infection and implies the proteolytic activity of caspase-5 and -10 in the generation of viral antigenic peptides recognized by CD8<sup>+</sup> cytotoxic T lymphocytes. More than 14 different members are included in the mammalian family of caspases (12), a kind of cytoplasmic cysteine proteases, some of which are involved in inflammatory responses, whereas others are essential for programmed cell death in a variety of species (12). They can be detected in many different types of cells undergoing apoptosis, regardless of their origin or the death stimulus, including infection by many pathogens (13, 14). Initiator caspases are the first to be activated, followed by effector caspases, which act on cellular target molecules. They are synthesized as zymogens with no or very low intrinsic enzymatic activity. Direct aggregation of initiator caspases after the triggering stimulus is sufficient to promote self-processing to generate active heterotetramers. Downstream caspases are then activated proteolytically by initiator caspases in a cascade fashion. When studied separately, two human initiator caspases, 5 and 10, processed the 19-mer encompassing the CMV epitope *in vitro*, with each generating a variety of peptidic products (9). We are interested in the study of possible cooperative effects of both cysteine proteases in the antigen processing of this mini-protein. This *in vitro* study shows qualitative as well as quantitative differences in the products obtained from single caspase digestions when compared with the mixture of both enzymes, indicating a concerted action of caspase-5 and -10.

## EXPERIMENTAL PROCEDURES

**Reagents**—Ac-WEHD-CHO, Ac-YVAD-CHO, and Ac-DEVD-CHO caspase inhibitors used in control digestions were purchased from the Peptide Institute (Osaka, Japan). m19 (MDIGAYPHFMPTNLAGDPY) peptide was synthesized in an Applera (Foster City, CA) peptide synthesizer model 433A, purified, and confirmed to be homogeneous by HPLC analysis. The sequence of the cytomegalovirus natural 9pp89 epitope is in boldface type. The nonamer 9pp89 with the sequence **YPH-FMPTNL** is the murine CMV immunodominant epitope presented by H-2L<sup>d</sup> (15).

**Caspase Digestion Assays**—Caspase-5 and -10 were expressed selectively in bacteria and purified as described (9, 16). m19 digestions by caspases were performed as follows (9, 16);

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TABLE 1

Digestion pattern of m19 synthetic peptide <sup>1</sup>MDIGAYPHFMPTNLAGDPY<sup>19</sup> with purified caspase-5 and/or -10 analyzed by mass spectrometry

Peptide sequence <sup>a</sup>	Correct C terminus	Residues <sup>b</sup>	Caspase-5	Caspase-10	Caspase-5 and -10
YPHFMPNTNL	Yes	6–14	0 <sup>c</sup>	0.74	0.41
AYPHFMPTNL	Yes	5–14	0	2.43	0.81
YPHFMPNTNLA		6–15	0	2.14	1.22
AYPHFMPTNLA		5–15	0.36	0	<b>20.00</b>
AYPHFMPTNLAG		5–16	0	1.00	0.30
YPHFMPNTNLAGD		<u>6–17</u>	0	0	<b>0.11</b>
AYPHFMPTNLAGD		<u>5–17</u>	0.66	1.32	0
DIGAYPHFMPTNLA		2–15	0	2.40	2.02
DIGAYPHFMPTNLAG		<u>2–16</u>	0.55	1.42	0
IGAYPHFMPTNLAGD		3–17	0.59	1.14	0.66
MDIGAYPHFMPTNL	Yes	<u>1–14</u>	1.19	0	<b>22.20</b>
YPHFMPNTNLAGDPY		6–19	0	68.19	36.95
MDIGAYPHFMPTNLA		1–15	0.50	0	2.36
DIGAYPHFMPTNLAGD		<u>2–17</u>	3.66	0	0
MDIGAYPHFMPTNLAGDP		<u>1–18</u>	0.79	0	0
<b>Total</b>			8.29	80.77	87.03

<sup>a</sup> Sequences of cleavage products were identified by mass spectrometry. The sequence of the cytomegalovirus natural 9pp89 epitope is in boldface.<sup>b</sup> The peptidic products increased in the digestion with the mixture of both caspases with respect to individual digestions are indicated in boldface. Peptides identified in individual digestions but not in the mixture of caspases are underlined.<sup>c</sup> Intensity of spectrum normalized to the average base peak ( $\times 10^6$ ). Data are means of three different experiments.

the m19 peptide, dissolved in dimethyl sulfoxide, was incubated overnight at room temperature with a concentration of 2  $\mu\text{M}$  of each caspase in 0.2 ml. When the mixture of both enzymes was used, identical amounts of each enzyme were added in the same final volume. Digestion time of 16 h was chosen to mimic time of exposure of m19 to proteases in infected cells (9). The final concentration of m19 was 0.5 mM, and the incubation buffer consisted of 0.1 M HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT. After incubation, the samples were frozen at  $-70^\circ\text{C}$  until further analysis. Similar experiments in presence of 0.2 mM WEHD-CHO or 2 mM YVAD-CHO (to inhibit caspase-5), or 0.2 mM DEVD-CHO (to block caspase-10 activity), were used as caspase specificity controls. Control digestions in the presence of caspase inhibitors did not reveal any cleavage product other than the m19 substrate (data not shown). The data are mean values of three independent experiments.

**MS**—Aliquots of total digestions were dried and dissolved in 10  $\mu\text{l}$  of 0.5% acetic acid in water and sequenced by quadrupole ion trap microHPLC (Biobasic C18 column 150  $\times$  0.18 mm, Thermo Fisher Scientific, San Jose, CA) electrospray MS/MS<sup>2</sup> in a Deca XP LCQ mass spectrometer (Thermo Fisher Scientific). The eluents used were as follows: A, 0.5% acetic acid in water; B, 80% acetonitrile containing 0.5% acetic acid. The gradient was 0–40% B in 24 min, 40–100% in 5 min, at a flow rate of 1.5  $\mu\text{l}/\text{min}^{-1}$ . We used the MS/MS mode focused to each hypothetical parental peptide with an isolation width ( $m/z$ ) of 1.5 Da (9, 17). The charge and the mass of the ionic 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. All identified peptides were sequenced by MS/MS fragmentation.

## RESULTS AND DISCUSSION

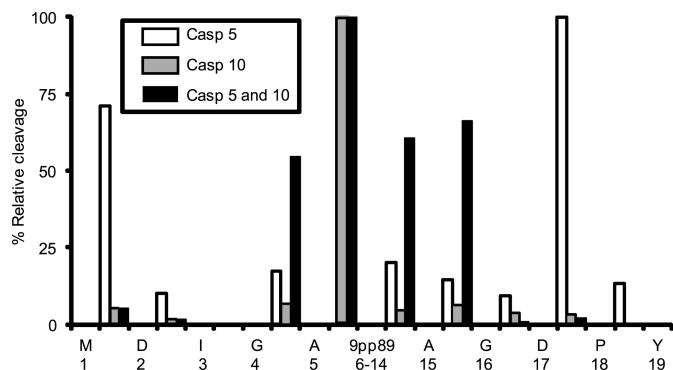
As a first step, caspase-5 and -10 were isolated and purified as reported previously (9, 16). Next, the m19 synthetic peptide was incubated with either caspase-5, caspase-10, or a mixture of both caspases. Each total digestion was analyzed extensively by

MS/MS to detect all relevant products, *i.e.* potential epitope precursors as well as the final epitope. Microliquid chromatography coupled to MS/MS was used, and the analysis was restricted to each theoretical parent ion and followed by manual interpretation (9, 17). This technique was employed as it increases peptide detection and optimizes the identification of generated peptides because it can resolve isomeric products generated from this miniprotein that include the CMV immunodominant epitope, such as A9pp89 and 9pp89A or GA9pp89 and 9pp89AG, as we reported previously in the analysis of antigen processing of this m19 synthetic peptide by proteasomes (17). Peptides such as DIGA9pp89AG and IGA9pp89AGD also have the same  $m/z$  value. In addition, as the m19 sequence presents two Met residues that are oxidized easily, located at the N terminus and at residue 10, all relevant processed peptides could also appear as the oxidized molecular ion. As a consequence, some peptides with oxidized Met such as DIGA9pp89AGD and other not oxidized ones, such as MDIGA9pp89AG, present identical  $m/z$  values but can be distinguished by this technique (17).

As shown in Table 1, caspase-5 or -10 produced a broad spectrum of processed peptides. As we reported previously (9), these products were unexpected given the canonical enzyme specificity. Caspase-5 or -10 generated either the <sup>2</sup>DIGAYPHFMPTNLAGD<sup>17</sup> or the <sup>6</sup>YPHFMPNTNLAGDPY<sup>19</sup> peptides as major cleavage products, respectively. The latter peptide exhibits the exact N-terminal Tyr of 9pp89 epitope. The second most abundant peptide produced by caspase-5 was the N-terminal extended peptide <sup>1</sup>MDIGAYPHFMPTNL<sup>14</sup>, which has the correct C-terminal Leu. Caspase-10 also produces low amounts of the exact optimally antigenic <sup>6</sup>YPHFMPNTNL<sup>14</sup> 9pp89 nonamer. In addition, several peptides were identified in these single caspase digestions. Lastly, the proteolytic efficiency of caspase-10 was 10-fold higher than that of caspase-5 (Table 1). Control digestions in the presence of caspase-5- or caspase-10-specific inhibitors did not reveal any antigenic peaks other than the m19 substrate (data not shown). This lack of other peptidic products in these control digestions, as well as the diversity of

<sup>2</sup> The abbreviations used is: MS/MS, tandem mass spectrometry.

## Concerted Antigen Processing by Caspases

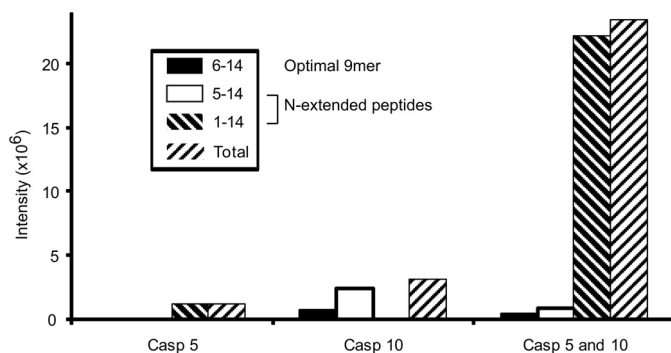


**FIGURE 1. Comparison of cleavage patterns of m19 synthetic peptide with purified caspase-5 and/or -10.** The m19 synthetic peptide was digested with the purified caspase-5 (*Casp 5*; open bars), caspase-10 (*Casp 10*; gray bars), or with a mixture of the two enzymes (*Casp 5 and 10*; filled bars) and analyzed by MS/MS as in Table 1. The bars indicate the relative frequency of each cleavage, deduced from the amounts of identified peptide products shown in Table 1. Amounts are depicted relative to the most abundant product within each digestion.

products generated by digestion with each enzyme, speaks for the purity of the caspase preparations.

When the mixture of both caspase-5 and -10 was used as enzymatic source, the <sup>6</sup>YPHFMPNLAGDPY<sup>19</sup> peptide, detected in the single caspase-10 digestion, also was the most abundant product (Table 1). Other cleavage products produced by the isolated activity of either caspase-5 or caspase-10 were also identified, including the optimal antigenic 9pp89 nonamer. Unexpectedly, a minor amount of peptide <sup>6</sup>YPHFMPNLAGD<sup>17</sup> was identified in the mixture of caspases but not in individual digestions with each single enzyme (Table 1, *boldface text*). Two additional cleavage products, <sup>1</sup>MDIGAYPHFMPN<sup>14</sup> and <sup>5</sup>AYPHFMPNLA<sup>15</sup>, were much more efficiently generated by the combined action of the two caspases than in the individual digestions (Table 1, *boldface text*). Lastly, four peptides found in caspase-5 digestions, two of which also were produced by caspase-10, were not detected in the experiment with the two caspases (Table 1, *underlined text*). In summary, the collection of peptidic products generated by the mixture of caspase-5 and -10 is different than the simple addition of individual caspase products.

To study the specificity of mixed *versus* single digestions the frequency of each cleavage was calculated. As shown in Fig. 1, cleavage of Met<sup>1</sup>–Asp<sup>2</sup> and Asp<sup>17</sup>–Pro<sup>18</sup> peptide bonds was preferred by caspase-5. In contrast, cleavage between Ala<sup>5</sup> and Tyr<sup>6</sup>, which is the C-terminal residue of the immunodominant 9pp89 epitope, was preferred largely by caspase-10 (Fig. 1). This was also the most prominent cleavage when the mixture of caspases was used. But in addition, significant cleavages around the immunodominant 9pp89 core (Gly<sup>4</sup>–Ala<sup>5</sup>, Leu<sup>14</sup>–Ala<sup>15</sup>, and Ala<sup>15</sup>–Gly<sup>16</sup>) were found abundantly in the mixture of caspases but almost not in each single digestion. Thus, the drop in the frequency of Met<sup>1</sup>–Asp<sup>2</sup> and Asp<sup>17</sup>–Pro<sup>18</sup> cleavage points typical of caspase-5, the increase of these three new cleavage points as well as the identification of the new product <sup>6</sup>YPHFMPNLAGD<sup>17</sup> in the mixed enzyme condition indicate a cooperative activity of both caspase-5 and -10 in antigen processing of the 19-amino acid-long peptide encompassing the CMV 9pp89 epitope.



**FIGURE 2. Comparative analysis of the generation of N-terminal extended precursors of the final size epitope.** The m19 synthetic peptide was digested with the purified caspase-5 (*Casp 5*), caspase-10 (*Casp 10*), or with a mixture of the two enzymes (*Casp 5 and 10*) and analyzed by MS/MS as in Table 1. The code used of detected products is as follows: 6–14 peptide (filled bars), 5–14 peptide (open bars), 1–14 peptide (thick hatched bars), and total (thin hatched bars).

In addition, some peptides having the correct C terminus and N-terminal extensions of the optimal 9pp89 nonamer were identified in either individual or combined caspase digestions (Table 1). These precursors could be susceptible to trimming by ERAP enzymes in living cells can thus be relevant for antigen presentation. This is especially important for the murine CMV pp89 immunodominant epitope, which must be transported to the ER lumen as N-terminal extended precursors because the final 9pp89 ligand is very inefficiently translocated by TAP (18). Small amounts of N-terminal extended precursors such as <sup>5</sup>AYPHFMPN<sup>14</sup> and <sup>1</sup>MDIGAYPHFMPN<sup>14</sup> were detected in individual caspase digestions (Fig. 2). After a combined caspase-5 and -10 exposure, the generation of <sup>1</sup>MDIGAYPHFMPN<sup>14</sup> peptide was enhanced greatly to turn into the second major product of digestion (Table 1 and Fig. 2). This resembled the activity of the proteasome on the 19-mer substrate (17, 19) and suggests that the combined activity of both enzymes could improve the efficiency of antigen presentation by the proteasome-independent caspase-mediated pathway reported previously (9).

Caspase-5 and -10 are both initiator caspases of proinflammatory (20) and apoptotic (21) pathways induced by vaccinia infection. Even though physical association has not been reported, our data suggest that they can use their respective degradation products as a substrate for new cleavages. Initial cooperation between separate enzymes in antigen processing was described for furin and amino- and carboxypeptidases in the secretory pathway (22). In addition, similar to our present report, a concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidases was required for the digestion of a 15-mer peptide with an amino-terminal extension of six residues (23), which is a natural epitope endogenously processed from the HIV gp160 glycoprotein (24). Thus, cooperative activity of related and even unrelated (10) enzymes may not be a rare event in antigen processing of specific epitopes, and its relevance must be evaluated in future studies with other epitopes.

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