Chapter 4

A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation.

Immunity

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Immunity
A Major Role for TPPII in Trimming Proteasomal Degradation Products for MHC Class I Antigen Presentation

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Summary

Intracellular proteins are degraded by the proteasome, and resulting peptides surviving cytoplasmic peptidase activity can be presented by MHC class I molecules. Here, we show that intracellular aminopeptidases degrade peptides within seconds, almost irrespectively of amino acid sequence. N- but not C-terminal extension increases the half-life of peptides until they are 15 amino acids long. Beyond 15 amino acids, peptides are exclusively trimmed by the peptidase TPPII, which displays both exo- and endopeptidase activity. Surprisingly, most proteasomal degradation products are handled by TPPII before presentation by MHC class I molecules. We define three distinct proteolytic activities during antigen processing in vivo. Proteasome-generated peptides relevant for antigen presentation are mostly 15 amino acids or longer. These require TPPII activity for further trimming before becoming substrates for other peptidases and MHC class I. The heterogeneous pool of aminopeptidases will process TPPII products into MHC class I peptides and beyond.

Introduction

The proteasome is the dominant cellular protease degrading intracellular proteins (Kloetzel, 2001; Rock et al., 1994), including many newly synthesized proteins (Reits et al., 2000; Schubert et al., 2000). Only a small fraction of these peptides is translocated by the transporter associated with antigen processing (TAP) into the lumen of the endoplasmic reticulum (ER), where they can bind newly synthesized MHC class I molecules (reviewed by Yewdell et al., 2003). The vast majority of generated peptides are however degraded by cytoplasmic peptidases before being able to interact with TAP (Fruci et al., 2003; Reits et al., 2003). In addition, peptides can be trimmed by the ER-associated aminopeptidase ERAAP or ERAP1 before binding to MHC class I molecules (Saric et al., 2002; Serwold et al., 2002; York et al., 2002), while other peptides are translocated back into the cytoplasm by the Sec61 complex and subsequently degraded (Koopmann et al., 2000; Roelse et al., 1994). MHC class I molecules usually bind peptides of nine amino acids with appropriate anchor residues (Rammensee et al., 1993). The TAP transporter also prefers to translocate peptides of around nine amino acids (Momburg et al., 1994a), although substrate peptides up to 40 amino acids can be translocated into the ER lumen as well (Koopmann et al., 1996). TAP shows minor specificity for peptide sequences (Momburg et al., 1994b), which is not unexpected since it has to provide different class I molecules with peptide epitopes.

In vitro experiments suggest that most peptides generated by the proteasome will probably have an incorrect size for direct class I loading (Kisselev et al., 1999; Toes et al., 2001). N-terminal extended epitopes can be trimmed to the correct size for MHC class I binding by cytoplasmic and ER luminal aminopeptidases, but most peptides will be completely recycled into free amino acids (Reits et al., 2003). Several cytoplasmic aminopeptidases and endopeptidases have been identified, including leucine aminopeptidase (LAP) (Benigna et al., 1998), tripeptidyl peptidase II (TPPII) (Geier et al., 1999; Tomkinson, 1999), thimet oligopeptidase (TOP) (Mo et al., 1999), bleomycin hydrolase (BH) (Bromme et al., 1996; Stoltze et al., 2000), and puromycin-sensitive aminopeptidase (PSA) (Johnson and Hersh, 1990; Stoltze et al., 2000). Overexpression of LAP (Reits et al., 2003) or TOP (York et al., 2003) reduces class I expression, suggesting a role for these peptidases in trimming peptides for MHC class I molecules. In addition, the ER-associated aminopeptidase is also involved in peptide generation for MHC class I molecules but also their destruction (Serwold et al., 2002; York et al., 2002). Since cells lack cytoplasmic carboxypeptidase activities (Reits et al., 2003), the proteasome should generate the correct C terminus of peptides for the MHC class I peptide binding groove (Cascio et al., 2001), unless endopeptidases exist. TPPII appears to be essential for the generation of a particular HIV epitope (Seifert et al., 2003) and exhibits endopeptidase activity in vitro (Geier et al., 1999; Seifert et al., 2003). These data suggest that various peptidases modify the pool of class I peptides. Although there may be specialization in location, substrate specificity, and amounts, their collective activity may determine the outcome of a MHC class I response, since more than 99% of the peptides are destroyed by peptidases (Princiotta et al., 2003; Reits et al., 2003). The few peptides escaping peptidase activity may represent a small pool of particular peptides that resist proteases due to amino acid sequence and peptide length. Alternatively, a nonselective process that degrades peptides at random may be operational with some inefficiency in the form of escaping peptides. This is, however, hard to determine on the basis of the few known in vitro-determined peptidase specificities.

Here, we have studied the specificity of the collective cytoplasmic peptidase activities using internally quenched peptides introduced into living cells. Peptides are rapidly degraded by aminopeptidases without dramatic sequence specificity. N-terminal but not C-terminal exten-
Figure 1. Detecting Peptidase Activity in Living Cells

(A) Principle of detecting peptidase activity. Internally quenched peptides are injected in cells. The emission of the fluorescein side chain (F) is absorbed by the quencher Dabcyl moiety (Q). Emission of fluorescein can only be detected when the two groups are spatially separated after degradation.

(B) Representative degradation curves of two peptides in vivo. Model peptide T[K-Dabcyl]-NKTER[C-Fluorescein]Y (black line) was microinjected (indicated by the arrow) in a single melanoma cell (Mel JuSo), and the appearance of fluorescence was monitored at 520 nm. This experiment was repeated with the same peptide with an N-terminal D amino acid (D-T)[K-Dabcyl]NKTER[C-Fluorescein]Y (gray line). The figure shows a merge of the two experiments. Fluorescence appears immediately after introduction in the cell and goes to completion.

Sequence Specificity of the Intracellular Peptidase Pool

Some aminopeptidases like LAP have defined substrate specificities (Turzynski and Mentlein, 1990). Variation in the amino acid sequence of the reporter peptide may therefore affect the degradation rate in vivo. This is important since an increased peptide half-life should theoretically correlate with a better chance to reach the ER lumen, resulting in an improved class I presentation. To examine whether the cytoplasmic peptidases collectively show sequence selectivity, we systematically varied amino acids positioned at the first, second, third, or last position within an internally quenched reporter peptide sequence (Figures 2A–2D). The variable amino acids (indicated as “X”) were representatives of every chemical group of amino acids. With the exception of the third amino acid position, the variable amino acids were positioned outside the quencher-fluorophore box (indicated by “K” and “C”). Peptide degradation always started immediately after its introduction in living cells by microinjection and went to completion. Surprisingly, the different amino acids hardly affected the peptide half-life (Figures 2A–2D), the $t_{1/2}$ of full peptide degradation is plotted), indicating that the collective pool of peptidases is able to efficiently degrade every peptide sequence almost irrespective of the type of amino acid at these positions. The differences in peptide half-life of the tested combinations are within a factor 2. The rate of degradation decreased only when the N-terminal amino acid was replaced by an unnatural D amino acid.
amino acid is not cleavable. In summary, the heterogeneous pool of cytoplasmic peptidases in cells efficiently digests peptides with no major sequence selectivity unless stereoisomeric D amino acids are introduced.

Cytoplasmic Peptidase Activity, Substrate Length, and Antigen Presentation by MHC Class I

Since the second amino acid of the original reporter peptide contained the quencher group, we repositioned the quencher molecule to the third position and extended the N terminus by one additional amino acid to examine the effect of amino acid variations at the second position. While again no major effect of the sequence variation was observed, the half-life of the reporter peptide increased (Figure 2D). Apparently the presence of two additional N-terminal amino acids reduced proteolytic access to the quencher-fluorophore reporter. To examine the relationship between peptide length and in vivo half-life, the reporter peptide was extended either at the C or N terminus with a repeat of amino acids again representing the various chemical groups (Figure 3). Even a further extension by 18 amino acids did not increase the short half-life of the N-terminal reporter sequence in vivo. Apparently, trimming aminopeptidases are degrading the first N-terminal amino acids of the reporter sequence at similar rates, irrespective of the length of the C terminus.

If correct, then addition of amino acids at the N terminus should increase the reporter’s half-life, as trimming aminopeptidases have to remove more amino acids before encountering the internally quenched reporter. Indeed, extending the N terminus up to three additional amino acids increased the half-life of the reporter sequence (Figure 3B). Surprisingly, further extension by 6, 11, or 18 additional amino acids did not lead to a further increase in half-life. To exclude that the presence of one or more proline residues in the N-terminal extension affected antigen presentation by MHC class I molecules, a HLA-A2-restricted influenza M epitope was expressed with N- or C-terminal extensions (Table 1) corresponding to the sequences used in the model peptides tested in Figures 3A and 3B. The various minigenes were cloned in a vector upstream of an IRES sequence followed by GFP to ensure cotranscriptional expression. These constructs were stably expressed in HLA-A2-expressing Mel JuSo cells and sorted for equal GFP expression

Figure 2. Sequence Specificity of the Collective Peptidase Activities in Living Cells

(A–D) 9-mer model peptides with systematic amino acid variations at position 1 (A), 3 (B), and 9 (C) were tested for in vivo degradation by Mel JuSo cells. The sequences are shown in every panel with K representing Lys-Dabcyl, C the Cys-fluorescein, and the enlarged and underlined amino acid X the variable amino acid. A 10-mer peptide was used to test the effect of variable amino acids at position 2 (Figure 2D). Kat position 2 was preceeded by F. The variations (introduced at position X) represent the different chemical groups of amino acids with, in addition, P as imino acid and G as the smallest amino acid. L represents the hydrophobic; D, acidic; K, basic; F, aromatic; and T, neutral amino acids. D-[Y] is the D amino acid T. Shown is the half-life in seconds for every peptide (±SEM).
Peptidase Activity in Living Cells

Figure 3. Substrate Length Specificity of the Collective Peptidase Activities.

(A) C-terminal extensions. The 8-mer reporter peptide sequence T[K-Dabcyl]NKTER[C-Fluorescein]F containing the quencher-fluorophore combination was C-terminally extended with 3, 6, 11, or 18 amino acids. The extension is a repeat of the different amino acids as tested in Figure 2. The peptides were introduced in living cells, and the half-life was determined. Half-life (± SEM) is depicted.

(B) N-terminal extensions. The reporter peptide Y[C-Fluorescein]RET[NN[K-Dabcyl]]T has the reverse sequence as the reporter used for the C-terminal extensions. Also, the sequence of the N-terminal extensions is mirrored. The reporter peptide was extended with 1, 3, 6, 11, or 18 amino acids, and the half-life was determined in cells (± SEM).

(C) The effect of N- or C-terminal extensions on HLA-A2-restricted antigen presentation of the influenza NP-epitope. Cells expressing both HLA-A2 and the influenza M-epitope, the epitope with one or three C-terminal amino acid extensions (C1 or C3), or the epitope with N-terminal extensions varying from 1 to 15 additional amino acids (N1 to N15) (see Table 1). Equal expression of the minigene was controlled through GFP expression from the same transcript. Activation of the influenza M epitope-specific, HLA-A2-restricted T cell clone was measured by IFN secretion after an overnight culture at different effector/target cell ratios. The experiments were performed in triplicate, and means (± SEM) are indicated.

before determining the T cell response. The influenza epitope with a C-terminal extension of one or three amino acids were not presented. Mel JuSo cells are apparently unable to generate the properly sized 9-mer influenza epitope in the absence of carboxypeptidase activity (Reits et al., 2003). Contrary to what may be expected, extending the N terminus of the influenza epitope with one or five amino acids did not increase presentation of influenza M epitope to specific T cells.

Table 1. Peptide Epitopes Expressed by Minigenes for CTL Assay

<table>
<thead>
<tr>
<th>Epitope</th>
<th>(M) GLIGFVFTL</th>
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<tr>
<td>Epitope (C = 1)</td>
<td>(M) GLIGFVFTL P</td>
</tr>
<tr>
<td>Epitope (C = 3)</td>
<td>(M) GLIGFVFTL PGL</td>
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<tr>
<td>Epitope (N = 1)</td>
<td>(M) D GLIGFVFTL</td>
</tr>
<tr>
<td>Epitope (N = 3)</td>
<td>(M) FKD GLIGFVFTL</td>
</tr>
<tr>
<td>Epitope (N = 5)</td>
<td>(M) GPFKD GLIGFVFTL</td>
</tr>
<tr>
<td>Epitope (N = 15)</td>
<td>(M) FKDLGPFKDLGPFKD GLIGFVFTL</td>
</tr>
</tbody>
</table>

Moreover, extending the N terminus with 15 amino acids resulted in a reduced presentation of the influenza epitope (Figure 3C). Since the sequence of the shorter extensions is repeated in the longest sequence, these data indicate that N-terminal extensions do not necessarily improve antigen presentation. The longest extension generates a minigene product of 28 amino acids and may be a substrate for the previously observed peptidase activity that is active on larger fragments and may generate, though less efficiently, this specific epitope.

To investigate the role of this peptidase in more detail, peptides of 27 amino acids with the reporter sequence of 27-mer peptide was degraded relatively slowly as expected for N-terminal-extended peptides. When the reporter was placed at the N terminus, fluorescence appeared almost immediately due to rapid separation of the quencher and the fluorescein group. However, when
Chapter 4

Figure 4. Characterizing Peptidase Activity for Longer Peptides

(A) Positional variations. The half-life of the 27-mer peptide shown in Figures 3A and 3B was compared with a 27-mer containing the reporter sequence in the middle. The half-life of the peptides was determined in living cells (±SEM).

(B) The effect of N-terminal modifications on degradation of the 27-mer peptide. Amino acids one or one and two were exchanged for D amino acids (shown in bold and underlined) in the 27-mer model peptide with the reporter in the middle (as in Figure 3D). Alternatively, the free N terminus of this peptide was blocked with naphthylsulfone (indicated by an asterisk). The peptides were introduced in living Mel JuSo cells and degradation measured. The half-life (±SEM) is depicted. No degradation of the N-terminally blocked peptide was observed within a period of 200 s.

The same fluorescent-quencher reporter sequence was placed in the center of the 27-mer peptide, degradation was as fast as when the reporter sequence was placed at the N terminus (Figure 4A). This is unlikely due to gradual N-terminal trimming, since peptides with only three to six amino acids N-terminally of the reporter sequence are degraded considerably slower than this peptide (Figure 3B). Surprisingly, a corresponding 18-mer peptide with the reporter sequence at the C terminus was degraded almost as fast as the 27-mer with the centered reporter sequence, suggesting that they are not substrates for trimming amino peptidases but that the 18- and 27-mer are possibly targeted by an endopeptidase that cleaves these peptides into large pieces, thereby separating the quencher and fluorophore. To examine the requirements for an unmodified N terminus, the 27-mer peptide with the reporter sequence in the middle was modified by either placing one or two D amino acids at the N terminus or by blocking the N terminus (Figure 4B). Replacing the first two amino acids by D amino acids did not influence the half-life of the reporter sequence. However, blocking the free N terminus prevented degradation of the 27-mer peptide in vivo. These data suggest that long peptides are handled by distinct peptidases that can remove larger pieces but require a free N terminus.

Long Peptides Are Selectively Degraded by TPPII

Since proteasomal activity may be involved in degrading large peptide fragments, we measured peptide degradation in the presence of proteasome inhibitor, but no change in peptide half-life was observed (data not shown). Another candidate might be the large peptidase complex TPPII, which has reported exo- and endopeptidase activities (Geier et al., 1999). To test whether TPPII was the peptidase specialized in the degradation of the long peptides, the inhibitor butabindide was used. Butabindide is a water-soluble and highly specific reversible and competitive inhibitor for TPPII (Breslin et al., 2002; Ganellin et al., 2000; Renn et al., 1998; Rose et al., 1996) and is a small indole-based structure with some inherent instability in solution (Breslin et al., 2002).

We tested the effect of butabindide on the degradation of a 20-mer internally quenched peptide microinjected into cells (Figure 5A). The addition of 10⁻⁴ M butabindide to the medium was sufficient to completely inhibit the degradation of the 20-mer peptide in living cells. Since cells were always microinjected under serum-free conditions, the effect of serum on the stability of butabindide was tested. Even small amounts of fetal calf serum prevented the inhibitory effect of butabindide (Figure 5B). To our knowledge, butabindide has not been successfully used in in vivo experiments before to inhibit peptide degradation by TPPII, which may be explained by the apparent inhibitory effect of serum on butabindide treatment. To test the stability of butabindide in our experimental system, the 20-mer internally quenched peptide was microinjected in cells (in serum-free medium) at various times after addition of the inhibitor at 10⁻⁴ M. Complete inhibition of peptide degradation was observed until ~1.5 hr after butabindide addition, after which peptide degradation was recovering (data not shown).

We subsequently measured the degradation rates of peptides, varying in size between 9 and 27 amino acids, by microinjecting these in cells cultured in the presence or absence of butabindide. The rate of degradation for peptides up to 15 amino acids was not significantly affected by inhibition of TPPII, although the N-terminally extended 15-mer peptide was degraded less efficiently under these conditions. Presentation of the 14-mer influenza epitope expressed from minigenes was not affected when cells were first stripped to dissociate most surface class I molecules by acid wash and cultured for...
Peptidase Activity in Living Cells

Figure 5. TPPII and the Generation of Peptides by TPPII in Living Cells

(A) The effect of serum on the stability of the TPPII inhibitor butabindide. Cells were cultured in the presence or absence of FCS while incubated with 100 μM butabindide. Subsequently, the 20-mer peptide T[K-Dabcyl]NKTERR[C-Fluorescein]YPGLDKFPGLDK was microinjected and degradation was measured.

(B) The effect of butabindide on the presentation of an expressed 14-mer peptide. HLA-A2-expressing Mel JuSo cells were transfected with a 14-mer peptide (N = 6; see Figure 3C). Cells were acid stripped followed by culture for 16 hr in the presence or absence of butabindide (refreshed every 60 min). Activation of the influenza M epitope-specific, HLA-A2-restricted T cell clone was measured by IFN-γ secretion after o/n culture at E/T ratio 1:1. The experiments were performed in 6-fold, and means (± SEM, corrected for background) are indicated.

(C) The effect of inhibition of TPPII on peptide degradation in living Mel JuSo cells. Peptides of different length with the reporter sequence at different positions (as indicated, for sequence see Figure 3) were microinjected in cells cultured under serum-free conditions and in the absence or presence of 100 μM butabindide. Degradation of the peptides was determined by fluorescence emission at 540 nm and the half-life of the peptides (± SEM) is depicted. D.N.O., degradation not observed within the time of analysis (200 s).

16 hr in the absence or presence of butabindide followed by the CTL assay (Figure 5B). This indicates that butabindide treatment did not inhibit synthesis, assembly, and transport of MHC class I molecules, nor presentation of small peptides. However, the degradation of peptides larger than 15 amino acids appeared to be critically dependent on TPPII activity (Figure 5C) irrespective of the position of the reporter sequence, since no degradation was observed (D.N.O.) in the presence of butabindide. Apparently, peptides longer than 15 amino acids can only be efficiently degraded by TPPII in vivo.

To test whether butabindide affected proteasome
degradation under these conditions, we cultured Mel JuSo cells in the absence or presence of the proteasome inhibitor MG132 or butabindide for 2 hr in serum-free medium. Accumulation of the proteasome substrate cyclin D1 was detected only after inhibiting the proteasome (Agami and Bernards, 2000) and not when cells were cultured in the presence of butabindide (Figure 6A). To confirm that the effects of butabindide were due to inhibition of TPPII, the peptide degradation experiments were repeated after transient downregulation of TPPII using a vector containing GFP and a siRNA construct directed against TPPII (Seifert et al., 2003). Two days after transfection, cells showed a downregulation of TPPII expression up to sixty percent (data not shown). Since TPPII is not fully downregulated, we measured peptide degradation under saturated conditions in vivo. This was determined after titration of “cold” 27-mer peptides (containing a quencher but no fluorophore) with constant amounts of quenched peptides to saturate TPPII activity. At three times molar excess of cold 27-mer peptide, an 8-fold reduction in rate of degradation of the quenched 27-mer peptide was observed. This concentration of competing cold peptides was coinjected with the internally quenched peptides in control and siRNA expressing cells. The 9-mer reporter peptide was rapidly degraded independent of TPPII saturation or additional inhibition by siRNA (Figure 6B). Due to competition with the cold 27-mer, the 16-mer, 20-mer, and 25-mer reporter peptides were slower degraded when compared to Figure 5C, but hardly any degradation was observed when microinjected cells expressed siRNA. Although slower, the 15-mer reporter peptide
could apparently still be degraded by other peptidases in siRNA-expressing cells (Figure 6B). Similar to the results obtained with butabindide treatment, specific downregulation of TPPII results in the inhibition of degradation of peptides longer than 15 amino acids.

**TPPII and the Generation of MHC Class I Peptides**

TPPII may be specialized in degrading large peptide fragments but at the same time it may also destroy and create antigenic peptides. To examine the importance of TPPII in the proteolytic pathway resulting in class I peptides, we first assayed the cleavage pattern of TPPII. Therefore, a series of 16-mer peptides was generated with the reporter sequence at the C terminus and with an increasing number of D amino acids at the N terminus (Figure 7A). Degradation of these peptides was inhibited by butabindide, implying that the peptides were handled by TPPII (data not shown). Proteases can only cleave the peptide bond between natural L amino acids and not between D and L or between D amino acids. Replacing the first or two N-terminal amino acids reduced the rate of degradation of the reporter segment, probably by affecting the exopeptidase activity of TPPII that can remove one to three amino acid parts from the N terminus (Tomkinson, 1999). However, no further decrease in the rate of degradation was observed when more L amino acids were replaced by D amino acids. Apparently, TPPII has two activities: one activity that removes small fragments from the N terminus and a second but slower activity that generates larger fragments. Since a fragment containing eight D amino acids has to be cleaved after the following L amino acid, TPPII is apparently able to generate 9-mer fragments (or longer).

To determine the relevance of TPPII in peptide generation for MHC class I molecules, both M cell JuSo cells and freshly isolated peripheral blood lymphocytes (PBLS) were stripped to dissociate most surface class I molecules by acid wash. The cells were subsequently cultured in the presence or absence of the proteasome inhibitor lactacystin, butabindide, or a combination of the two inhibitors, all under serum-free conditions. Butabindide was readded every hour. After 4 hr, MHC class I molecules at the cell surface were labeled with the antibody W6/32, followed by FACS analysis (Figure 7B). Surprisingly, butabindide treatment resulted in a similar reduction in cell surface MHC class I as treatment with proteasome inhibitor, while the combination of the two inhibitors only had a marginal additive effect. Identical effects were obtained with the less specific TPPII inhibitor AAF-CMK (data not shown). Given the substrate specificity of TPPII, our data suggests that a major portion of proteasomal products consists of long peptides (over 15 amino acids) that are subsequently targeted by TPPII. Since these peptides will be too long for MHC class I loading, TPPII will act as a crucial intermediate between proteasomes, cytoplasmic or ER peptidases, and MHC class I molecules.

**Discussion**

The efficiency of antigen presentation by MHC class I molecules is amazingly low, since more than 99% of the peptides generated by the proteasome are lost before encountering the peptide transporter TAP (Fruci et al., 2002; Princiotta et al., 2003; Reits et al., 2003). These peptides are degraded within seconds by cytoplasmic peptidases and most fail to associate with TAP within this time window (Reits et al., 2003). A number of cytoplasmic peptidases have been characterized that may contribute to peptide processing for MHC class I presentation. For example, LAP is a peptidase that is upregulated by IFNγ and able to degrade peptides destined for antigen presentation (Beninga et al., 1998; Reits et al., 2003). TOP is another peptidase known to affect the class I peptide pool (Seric et al., 2001) and MHC class I molecules are upregulated when TOP is inactivated by siRNA (York et al., 2003). TPPII is reported to be involved in the generation of a HIV epitope (Seifert et al., 2003) and may generate C termini of epitopes that are not made by the proteasome. It has been suggested that TPPII can compensate for some activities of the proteasome (Glas et al., 1998) but how, if at all (Princiotta et al., 2001), is unclear. The specificity of the cytoplasmic peptidases may be an important determinant in the outcome of MHC class I antigen presentation if particular peptide sequences would be more resistant. Although the substrate specificity of some peptidases is known, it is unknown whether the combined in vivo activities and enzyme concentration destroys different cytoplasmic peptides equally.

Here, we show that variation of amino acids at the first, second, third, or last position does not result in major differences in the half-life of introduced peptides. Although the quencher and/or fluorophore might affect the ability of some peptidases to hydrolyse the reporter peptide, our data suggests that the heterogeneous pool of cytoplasmic peptidases destroys small peptides rapidly but fairly irrespective of amino acid sequence. Since peptides are degraded exclusively by aminopeptidases (Reits et al., 2003), additional N-terminal amino acids should increase the half-life of an antigen, as has been suggested previously (Casio et al., 2001). The half-life of the 9-mer reporter sequence increases from 4 to 30 s when three additional amino acids are present at the N terminus. Apparently, every additional amino acid increases the reporter’s half-life with around 6 s until the peptide becomes longer than 15 amino acids. Still, when ninhydrin constructs with N-terminal extensions are exposed, no increased level of inflammation antioxidant pre-sentation is observed. In fact, a long N-terminal extension (15 additional amino acids) results in decreased levels of antigen presentation. This may be surprising, since the short N-terminal sequences were included in the long N-terminal sequence, but it suggests that the antigenic sequence within the long peptide is partially degraded by an endopeptidase. Extending the total length of the reporter peptide beyond 15 amino acids does not reduce the rate of degradation any further, which suggests that these peptides were handled differently from shorter peptides by the cytoplasmic aminopeptidases. Extending the C terminus of the NP-epitope prevents presentation by MHC class I, which confirms the observed absence of cytoplasmic carboxypeptidase activities (Reits et al., 2003).

The enzyme involved in long peptide degradation should display aminopeptidase activity, since blocking
Figure 7. Major Role for TPPII in Antigen Presentation

(A) TPPII and the generation of 9-mer peptides. A 16-mer peptide was synthesized with the reporter sequence Y[C-Fluorescein]RETKN [K-Dabcyl]T at the C terminus. Either one, two, five, or eight N-terminal amino acids were exchanged for their corresponding D stereoisomers that cannot be handled by natural proteases. The peptides were introduced in living cells, and degradation of the reporter sequence was measured.

(B) TPPII and the generation of peptides for MHC class I molecules. Mel JuSo cells (left) and human PBLs (right) were acid stripped followed by culture in the presence or absence of lactacystin, butabindide, or a combination of the two inhibitors. Butabindide was refreshed every 60 min. After 4 hr, the surface expression of MHC class I molecules was determined by FACS analysis. The inhibitors used are indicated in the figure.

(C) Model of the first proteolytic steps in MHC class I antigen presentation. The proteasome releases mainly products of 16 amino acids or longer. These long peptides are handled almost exclusively by TPPII. TPPII can destroy but also generate antigenic peptides for TAP by either removing small packages of amino acids or making large fragments, thereby creating the C terminus. Smaller peptides can be produced directly by the proteasome and after further trimming by TPPII. Other peptidases (represented as X, Y, and Z) that include TOP and LAP target these substrates. Antigenic peptides translocated by TAP can be generated exclusively by the proteasome, by the proteasome and TPPII, and by the proteasome, often TPPII, and other peptidases. TPPII appears to be an important intermediate between the proteasome and the rest of the peptidase pool involved in trimming fragments to antigenic peptides and mainly free amino acids.
the N terminus also protects the long peptides. Thimet oligopeptidase has been shown in vitro to select peptide substrates between 8 and 16 amino acids in length (Saric et al., 2001), but it is unclear whether it can degrade longer peptides as well. TPPII is a peptidase that is even larger than the proteasome (5–9 MDa) and is able to generate antigenic fragments by endopeptidase activity (Geier et al., 1999). Although TPPII can degrade small trimer substrates in vitro (Geier et al., 1999), it is unclear whether this reflects the activities of the complex in vivo. Regulating subunits may not be copurified with the TPPII complex, leading to a situation where the 20S core is purified without the 19S subunits. TPPII is upregulated when various activities of the proteasome are inhibited (Geier et al., 1999; Princiotta et al., 2001), which can be envisaged when under these conditions the proteasome is generating larger breakdown intermediates requiring further fragmentation by TPPII. Alternatively, TPPII could replace the proteasome but should then be able to unfold and deubiquitinate substrates. Whether this is possible is unknown. TPPII is at least unable to degrade the proteasomal substrate cyclin D1. When we repeated the peptide degradation experiments in the presence of the specific TPPII inhibitor butabindide or after downregulation of TPPII by siRNA, we observed that TPPII was selectively involved in the degradation of peptides of over 15 amino acids, whereas the degradation of smaller peptides was not affected. Substrates with the reporter located nine amino acids from the N terminus were very efficiently degraded, suggesting that TPPII cleaves somewhere between the ninth and eleventh residue. Experiments with D amino acid-containing substrates suggest that TPPII can remove blocks of one to three amino acids for the N terminus but also fragments of nine amino acids or more (albeit less efficient). This corresponds to the reported in vitro activities of TPPII, where TPPII digests proteins mainly in fragments larger than 15 amino acids with two-thirds too short to function in antigen processing (Kisselev et al., 1999). However, it is likely that peptides reenter the proteasome multiple times during in vitro digestion, possibly resulting in shorter products than generated in vivo. Peptides are no substrate for the proteasome in living cells (Reits et al., 2003), and reentry into the proteasome will not be a relevant route in vivo. Our data imply that a major portion of proteasomal products is larger than 15 amino acids and require further degradation by TPPII before becoming relevant for MHC class I. It has been suggested that the proteasome usually generates the proper C terminus of class I binding peptides (Gascio et al., 2001) with aminopeptidases only trimming from the N-terminal side. Our data propose a role for TPPII in this process since TPPII can generate at least 9-mer (antigenic) fragments containing a new C terminus. TPPII may thus generate but also destroy antigenic peptides, which may explain why a long mingeine-expressed peptide is less efficiently presented than mingeine-expressed peptides shorter than 15 amino acids.

By visualizing peptidase activity in living cells, we have defined TPPII as a critical player in antigen processing and presentation by MHC class I molecules. Our data suggest the following sequence of events to occur for a successful class I response (Figure 7C). The proteasome digests proteins mainly in fragments larger than 15 amino acids. TPPII trims these peptides into smaller fragments that may include proper class I binding peptides. A variety of peptidases (including TOP, LAP, and ERAAP) then continues the digestion into even smaller products. These three proteolytic steps act in a process degrading proteins down to small fragments and amino acids, occasionally generating a class I binding peptide instead of destroying it. In every step, peptides may escape further trimming by cytoplasmic peptidase activity by being transported into the ER by TAP for consideration by ERAAP and MHC class I. Most peptides will however be degraded into single amino acids.

Experimental Procedures

Synthetic Peptides
The various fluorescent peptides were synthesized by solid-phase strategies using an automated multiple peptide synthesizer (Syro II, MultiSyntech, Witten, Germany) using Fmoc chemistry. Fluorescein was covalently coupled to the cysteine residue using fluorescein-5-iodoacetamide (Molecular Probes, Leiden, The Netherlands). Fmoc-L-Lys(Dabcyl)-OH was obtained from Neosystems (France). All peptides were HPLC purified (>95% pure) and validated by mass spectrometry.

Peptide Degradation Analysis
Cells on coverslips were placed on an inverted Zeiss Axiovert 135 microscope equipped with a dry Achroplan 63x (NA 0.75) objective.
Peptides were quantified by their red appearance (Dabcyl) using photospectrometry and mixed with Fura Red (Molecular Probes, Leiden, The Netherlands) as a microinjection marker. Excitation of both fluorescein and Fura Red was at 475 nm. Emission of fluorescein and Fura Red was measured after splitting the emitted light using a 580 nm dichroic mirror and simultaneously detected with PTI model 612 analog photomultipliers. For data acquisition, FELIX software (PTI Inc., USA) was used. Fura Red was used as a control for microinjection and cell leakage. The relative fluorescence of fluorescein was expressed as the ratio of fluorescein to Fura Red signal, as described (Reits et al., 2003). To inhibit proteasomal activity, cells were incubated in the presence of 10 μM lactacystin for 30 min at 37°C. To inhibit TPPi activity, cells were cultured at 37°C in serum-free HEPES-buffered Iscoves medium in the presence of 100 μM butabindide-oxalate (Tocris, Avonmouth, UK) dissolved in 10 mM Tris-HCl (pH 8.0) prior to immediate use. Microinjection of the internally quenched peptides was performed within 1.5 hr after addition of butabindide. Peptide half-life was determined in at least three independent experiments (generally at least five successful microinjections per experiment) and depicted as mean value ± SEM. The siRNA-targeting TPPi (5’-AAGTGGCAGATGGAATCTGCT-3’) (Seifert et al., 2003) was cloned into the vector pSUPER (Brummelkamp et al., 2002) containing GFP (kindly provided by R. Schotte and H. Spits). Cells were transiently transfected with the siRNA construct, and 3 days upon transfection cells expressing GFP were microinjected with a mixture of quenched peptides of different lengths in combination with three times the amount of nonfluorescent 27-mer peptides (to measure TPPi activity under saturated circumstances).

**FACS Analysis**

Peptides were stripped of surface MHC class I molecules on cells by mild acid treatment as described (Sugawara et al., 1987). In brief, cells were incubated on ice for 10 min, washed twice with cold medium (Iscoves’s medium without phenol red), incubated for 2 min with ice-cold citric acid-Na2PO4 buffer, followed by two washes with ice-cold Iscoves medium. Subsequently, cells were cultured at 37°C in medium alone or in the presence of 100 μM lactacystin, 10 μM lactacystin, or a combination of both inhibitors. Butabindide was replaced every hour, and after 4 hr cells were harvested, put on ice, and stained with FITC-conjugated W6/32. Fluorescence was measured by flow cytometry (Beckton Dickinson).

**Western Blotting**

MelJuSo cells were cultured in the absence or presence of 10 mM MG132 or 100 μM butabindide-oxalate for 2 hr before analysis of cell lysates by 10% SDS-PAGE and Western blotting. MHC class II molecules were detected by the mAb 1B5 and cyclin D1 by the Ab M-20.

**CTL Assay**

MelJuSo cells were stably transfected with HLA-A*0201 and pCDNA3 containing a minigene-IRES-GFP sequence. Cells were FACS sorted for equal HLA-A2 and GFP expression. Cells were cultured in the presence or absence of butabindide. The human T cell clone (Nov A 13 TGA) recognizing the Flu-MuMT1 peptide (GILGFVFTL) in the context of HLA-A*0201 was cultured n/w with target cells at various E:T ratios in the presence of IL-2 (20 IU/ml). The amount of IFNγ secreted by the T cells was detected in the supernatant by Elisa according to the manufacturer’s protocol (Sanquin, Pels paap reagent set human IFNγ, M9333). Controls for spontaneous IFNγ release and recognition of the specific peptide were included.

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**References**


peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. Immunity 13, 117–127.


