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Splicing of antigenic peptides by the proteasome

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Abbreviations

AAA ATPase	ATPase associated with diverse cellular activities
AEP	Asparaginyl endopeptidase
β 2m	Beta2-microglobulin
BH	Bleomycin hydrolase
BrAAP	Branched amino acid preferring
ConA	Concanavalin A
CP	Catalytic particle
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DRiP	Defective ribosomal product
DUB	Deubiquitylating enzyme
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum-associated aminopeptidase
ERAD	Endoplasmic reticulum-associated degradation
FGF-5	Fibroblast growth factor-5
HC	Heavy chain
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
IDE	Insulin-degrading enzyme
IFN γ	Interferon-gamma
IP	Immunoproteasome
IRAP	Insulin-regulated aminopeptidase
LAP	Leucine aminopeptidase
LMP2/7	Low molecular weight protein 2/7
MECL1	Multicatalytic endopeptidase complex-like 1
MHC	Major histocompatibility complex
MS	Mass spectrometry
Nf- κ B	Nuclear factor-kappaB
PGPH	Peptidyl-glutamyl peptide hydrolysing
PLC	Peptide-loading complex

PNGase	Peptide- <i>N</i> -glycanase
PSA	Puromycin-sensitive aminopeptidase
RP	Regulatory particle
Rpn	Regulatory particle non-ATPase
Rpt	Regulatory particle ATPase
siRNA	Small interfering RNA
SNAAP	Small neutral amino acid preferring
SP	Standard proteasome
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TNF α	Tumor necrosis factor-alpha
TOP	Thimet oligopeptidase
TPPII	Tripeptidyl peptidase II
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system

Summary

Antigens presented by MHC class I molecules to CD8⁺ T lymphocytes consist of peptides of 8-11 amino acids, corresponding to continuous fragments of intracellular proteins. However, some antigenic peptides were found to be created by the splicing of two distinct fragments of the respective parental proteins. Peptide splicing represents a newly described mode of production of antigenic peptides whereby two noncontiguous peptide fragments are joined together after the excision of an intervening segment. Three spliced antigenic peptides have been described. The first example is derived from fibroblast growth factor-5 (FGF-5) and is made up of two fragments of five and four residues. Another is a peptide produced from melanosomal protein gp100 by splicing of two fragments of three and six residues. The third example is a human minor histocompatibility antigenic peptide created by a polymorphism in the SP110 gene. This epitope is composed of two noncontiguous fragments of four and six residues which are spliced together in the reverse order to that in which they appear in the parental protein. In the two latter cases, splicing involves the excision of a short intervening segment of four or six residues and was shown to occur in the proteasome by transpeptidation resulting from the nucleophilic attack of an acyl-enzyme intermediate by the N terminus of a peptide fragment present at the active site.

In a first part of this work, we studied the mechanism of production of the FGF-5 spliced peptide, for which the length of the intervening segment (40 aa) is much longer than the two other described examples. We confirmed that the proteasome also produces this spliced peptide by transpeptidation and we showed that reducing the length of the intervening segment increased the production of the spliced peptide. We also tested the possibility of *trans*-splicing (i.e., splicing of fragments from two distinct substrates) and observed that this event can occur in the cell between two peptide precursors with the same efficiency as *cis*-splicing. However, *trans*-splicing between two distinct proteins was far less efficient than splicing of peptide fragments from a single protein substrate and unlikely contributes to the production of the spliced peptides derived from FGF-5 and gp100.

The production of the three spliced peptides by the standard proteasome and the immunoproteasome was then compared in details, using a cellular approach and an in vitro approach. We showed that both proteasome types can produce spliced peptides although they differ in their efficiency of production of each peptide. The FGF-5 and gp100 peptides are produced more efficiently by the standard proteasome, while the SP110 peptide is better

produced by the immunoproteasome. By showing that splicing depends on the efficiency of production of the splicing partners, these results support the transpeptidation model of peptide splicing.

In collaboration with Dr. Paul Robbins from the National Cancer Institute in Bethesda, we have identified a new spliced antigenic peptide, which is derived from the melanosomal protein tyrosinase. This original peptide is composed of two spliced and rearranged peptide fragments, each one containing an aspartate residue resulting from asparagine deamidation. The processing of this antigenic peptide involves retrotranslocation of tyrosinase molecules from the endoplasmic reticulum (ER) to the cytoplasm, deamidation of presumably glycosylated asparagines by peptide-*N*-glycanase, cleavage and splicing by the standard proteasome, and transport of the resulting peptide into the ER via the TAP transporter. The potential role of an additional, yet unidentified, protease during this process is also discussed in this work.

Résumé

Les lymphocytes T CD8 reconnaissent des peptides de 8 à 11 acides aminés qui sont présentés à la surface cellulaire par les molécules du complexe majeur d'histocompatibilité de classe I. Généralement, ces peptides correspondent à des fragments continus des protéines cellulaires dont ils proviennent. Cependant, des travaux récents ont montré que certains peptides antigéniques pouvaient correspondre à la fusion de deux fragments non-contigus de la protéine parentale. Ce phénomène, appelé l'épissage peptidique, représente un nouveau mode de production de peptides antigéniques par lequel deux fragments peptidiques qui ne sont pas contigus, sont assemblés après l'excision d'un fragment intermédiaire. Trois peptides antigéniques épissés ont été décrits à ce jour. Le premier provient de la protéine FGF-5 et est composé de deux fragments, un de cinq et l'autre de quatre acides aminés. Un autre peptide épissé est produit à partir de la protéine mélanocytaire gp100 par l'épissage d'un fragment de trois acides aminés avec un autre de six acides aminés. Le troisième exemple est un antigène mineur d'histocompatibilité créé par un polymorphisme au sein du gène SP110. Cet épitope est composé de fragments non-contigus de quatre et six acides aminés qui sont assemblés en orientation inverse par rapport à la séquence de la protéine SP110. Pour les deux derniers peptides, l'épissage nécessite l'excision d'un court fragment intermédiaire de quatre ou six acides aminés et a lieu à l'intérieur du protéasome. Le mécanisme proposé correspond à une transpeptidation qui découle de l'attaque nucléophile d'un intermédiaire acyl-enzyme par le groupement N-terminal d'un fragment peptidique présent au niveau du site actif.

En premier lieu, nous avons étudié le mécanisme de production du peptide FGF-5, pour lequel la taille du fragment intermédiaire (40 aa) est beaucoup plus longue que dans le cas des deux autres peptides épissés. Nous avons confirmé que le protéasome est aussi responsable de la production de ce peptide épissé, et ce par le même mécanisme de transpeptidation. Nous avons aussi montré que le fait de réduire la longueur du fragment intermédiaire permet d'augmenter la production du peptide épissé. La possibilité que deux fragments issus de deux substrats distincts soient épissés ensemble a aussi été testée. Nous avons observé que ce phénomène d'épissage "en *trans*" pouvait être aussi efficace que l'épissage "en *cis*" dans les cellules à partir de substrats peptidiques. Cependant, l'épissage "en *trans*" à partir de deux protéines distinctes paraît beaucoup moins efficace que l'épissage entre deux fragments provenant de la même protéine, et ne semble pas contribuer à la production des peptides épissés issus de FGF-5 et gp100.

Nous avons ensuite comparé la production des trois peptides épissés entre le protéasome standard et l'immunoprotéasome, et ce en utilisant une approche cellulaire et une approche *in vitro*. Ceci nous a permis de vérifier que les deux types de protéasomes sont capables de produire des peptides épissés, bien que leur efficacité diffère pour produire chacun d'eux. Les peptides FGF-5 et gp100 sont mieux produits par le protéasome standard, alors que le peptide SP110 est mieux produit par l'immunoprotéasome. En montrant que l'efficacité d'épissage dépend de la capacité à produire les fragments qui doivent être épissés, ces résultats étayent d'une certaine manière le modèle de transpeptidation.

En collaboration avec le Dr. Paul Robbins du National Cancer Institute à Bethesda, nous avons pu identifier un nouvel épitope épissé provenant de la protéine mélanocytaire tyrosinase. L'originalité de ce peptide antigénique repose sur le fait qu'il est composé, d'une part de deux fragments épissés en orientation inverse, et d'autre part que chacun des deux fragments contient un acide aminé aspartate qui provient de la déamidation d'une asparagine. L'apprêtement de ce peptide implique la rétrotranslocation de la protéine tyrosinase du réticulum endoplasmique (RE) vers le cytoplasme, la déglycosylation/déamidation des asparagines par la peptide-*N*-glycanase, le clivage de la protéine et l'épissage par le protéasome standard, puis le transport du peptide obtenu vers le RE grâce aux transporteurs TAP. Le rôle potentiel d'une deuxième protéase, non encore identifiée, est aussi discuté dans ce travail.

INTRODUCTION

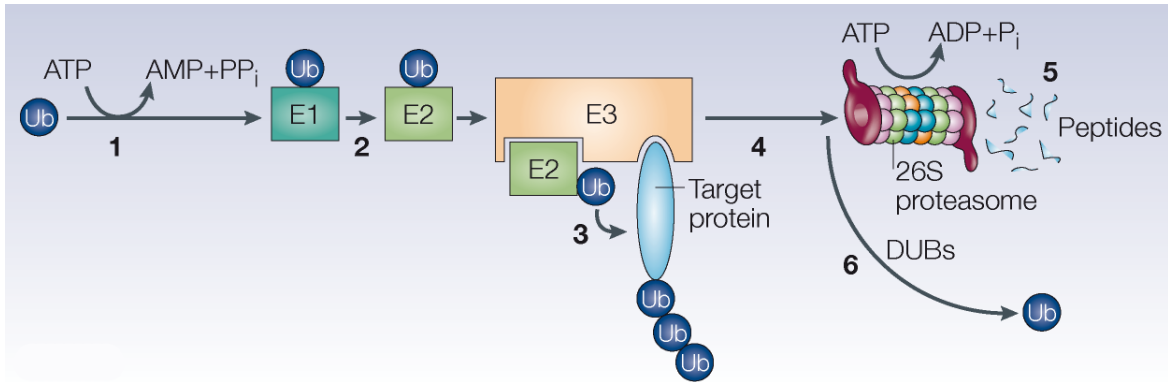


Figure 1. Ubiquitin conjugation and the ubiquitin–proteasome system.

Ubiquitin (Ub) is activated by the ubiquitin-activating enzyme (E1; *step 1*), and is subsequently transferred to a ubiquitin-conjugating enzyme (E2; *step 2*). In most cases, the E2 enzyme and the protein substrate both bind specifically to a particular ubiquitin-protein ligase (E3), and the activated ubiquitin moiety is then transferred to the protein substrate (*step 3*). The successive conjugation of ubiquitin moieties generates a polyubiquitin chain that functions as a signal to target the protein substrate to the 26S proteasome for degradation (*step 4*). The substrate is degraded into short peptides (*step 5*), and reusable ubiquitin is released by deubiquitylating enzymes (DUBs; *step 6*) (25).

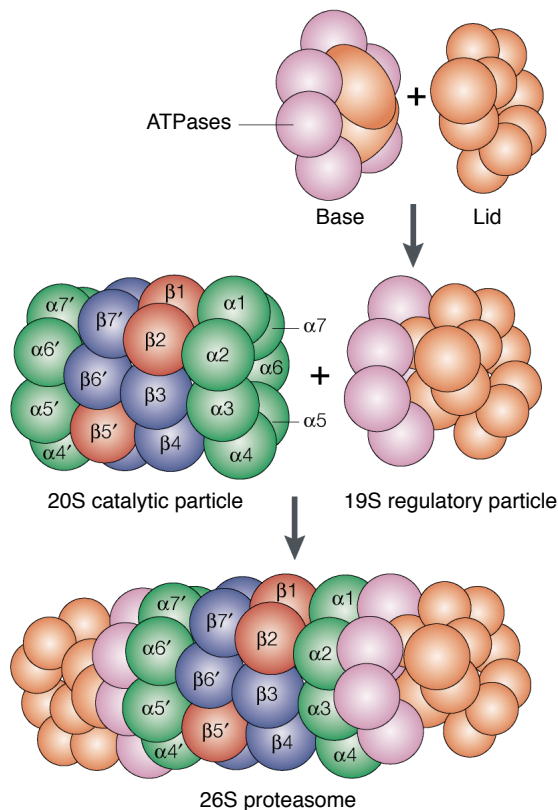


Figure 2. Composition of the 26S proteasome.

The eukaryotic 26S proteasome is composed by the 20S catalytic particle bound at both ends with two 19S regulatory particles. The 20S catalytic particle (20S proteasome) consists of 28 (14 different) subunits arranged as four heptameric stacked rings. The two outer rings contain the α -subunits ($\alpha 1$ – $\alpha 7$, *green*) and the two inner rings contain two copies of the β -subunits ($\beta 1$ – $\beta 7$; *blue and red*). Three of them ($\beta 1$, $\beta 2$ and $\beta 5$; *red*) harbour the six active sites. The 19S regulatory particle comprises two substructures. The base, which attaches to the two α -rings, is composed of six ATPase (*purple*) and four non-ATPase subunits (*orange*). The lid contains at least nine non-ATPase subunits and possess a deubiquitylating activity (10).

I) Degradation of cellular proteins by the ubiquitin-proteasome system

The concept of intracellular protein degradation emerged in the middle of the 20th century when Rudolph Schoenheimer showed, with the use of heavy isotopes, that protein components of the body were continuously synthesized and degraded (1). A few years later, Christian de Duve discovered the lysosomes, an intracellular organelle containing proteases with different specificities (2-4). For the first time, a machinery that could mediate intracellular protein degradation was identified. During the following twenty years, accumulating evidence indicated that the lysosome was not involved in the degradation of most intracellular proteins under basal metabolic conditions. Hence, it was supposed that the cells contain two different proteolytic systems: the lysosome that degrades principally extracellular proteins and another system that degrades most intracellular proteins (5, 6). The existence of a non-lysosomal proteolytic system was also supported by the observation that degradation of cellular proteins requires metabolic energy (7), despite the exergonic nature of peptide bond cleavage, what was in contrast with the known mode of action of lysosomal proteases. Etlinger and Goldberg were the first to isolate a cell-free preparation from reticulocytes, presenting a non-lysosomal and an ATP-dependent proteolytic activity (8). This led to the discovery of the ubiquitin signalling system and the proteasome in the 1980's.

The ubiquitin-proteasome system (UPS) represents the major pathway for intracellular degradation of soluble proteins in eukaryotes. This ATP-dependent system degrades about 80% of intracellular proteins and, thereby, is responsible for basal protein turnover and cellular homeostasis (9). The proteasome is a multi-catalytic complex found both in the nucleus and in the cytoplasm. This proteolytic machinery degrades, in most cases, substrates that have been tagged with ubiquitin (Ub). The two major steps of this system are the conjugation of Ub chains to the substrates and their subsequent proteolytic cleavage (**Figure 1**). They are tightly coordinated by the main components of the system, which are the ubiquitylation enzymes and the different proteasome particles. The generation and the covalent attachment of polyubiquitin chains requires the activity of three classes of enzymes: E1, E2 and E3. These polyubiquitin chains serve as a recognition motif for the 19S regulatory particle (RP), which is part of the 26S proteasome (**Figure 2**) (10). Also, the 19S RP is responsible for the unfolding of the substrate and its translocation into the 20S catalytic particle. The substrate is further degraded by the catalytic activities of the 20S proteasome into peptides of various lengths. The UPS is not limited

to purely metabolic functions. By degrading mainly short-lived proteins, it contributes to the protein quality control of the cells by eliminating misfolded or abnormal proteins (11), and regulates many cellular functions such as cell cycle progression and apoptosis (12). This system also participates in the degradation of several transcription factors and proteins implicated in oncogenesis (13), but is also responsible for the activation of the transcription factor NF- κ B, which has a central role in immune and inflammatory responses (14, 15). Importantly, the UPS plays a major role in cellular immunity by supplying most of the antigenic peptides presented by major histocompatibility complex (MHC) class I molecules (16).

1. Ubiquitylation machinery

The first step in the UPS, before peptide bond cleavage by the proteasome, is the covalent modification of the substrate with polyubiquitin chains. Ubiquitin (Ub) is a small protein of 76 amino acids expressed in cells from all eukaryotes, with a highly conserved primary structure. Its principal role is to tag the proteins that will undergo proteasomal degradation but many other purposes for ubiquitin conjugation were also described.

The role of ubiquitin in protein degradation and the mechanism of ubiquitylation was discovered by Hershko, Ciechanover and colleagues (17-19). Three different classes of enzymes, E1, E2 and E3, are involved in the cascade reaction of Ub conjugation (**Figure 1**). The Ub-activating enzyme E1, activates Ub in an ATP-dependent manner. Ub is then transferred to an Ub-conjugating enzyme E2. The E2 enzyme and the protein substrate both bind specifically to a Ub-protein ligase E3, which presents the substrate to E2 for Ub conjugation. Thereby, Ub is linked via its C terminus to the ϵ -amino group of a lysine residue of the substrate via an isopeptide bond. For accurate selection of the protein substrates, a large variety of enzymes is mobilized. In humans, more than 500 different species of E3 enzymes have been identified. Each of them usually selectively recognizes a protein substrate for ubiquitylation. Serial attachment of additional Ub molecules to form polyUb chains occurs by repetitive passage through the E1-E2-E3 cycle, with the formation of an isopeptide bond involving Lys48 of the initial Ub molecule and C terminus of the new one. PolyUb chains can also be assembled by E4 enzyme before attachment to the substrate (20). A chain of at least four Lys48-linked Ub molecules is sufficient for recognition and degradation of a protein substrate by the proteasome (21). However, many studies have shown that some substrates can also be degraded by the proteasome without attachment of Ub molecules; reviewed in (22). This Ub-independent proteasomal degradation

seems to have been underestimated and presents an increasing importance, notably since the identification of a number of oncoproteins and oncosuppressive proteins as proteasomal substrates degraded independently of ubiquitylation.

On the contrary, the attachment of ubiquitin does not always serve as a proteasomal degradation signal. Polyubiquitylation can be involved in DNA repair (23), while monoubiquitylation is principally involved in histone regulation, endocytosis and the budding of retroviruses from the plasma membrane (24). Interestingly, after endocytosis, mono- or oligo-ubiquitylation (chains of two or three Ub) can target membrane proteins for degradation in the lysosomes (25). Thus, ubiquitylation can also serve as a degradation signal for autophagy. However, in the cases of DNA repair and lysosomal targeting, protein substrates are modified with Lys63-linked Ub chains and not with Lys48-linked Ub chains.

Ubiquitylation is a reversible reaction. The removal of Ub from proteins and the disassembly of Ub chains is called deubiquitylation and is catalysed by deubiquitylating enzymes (DUB), also called isopeptidases (26). These enzymes provide additional regulatory control before protein degradation and are essential for maintaining a sufficient pool of free Ub molecules in the cell. Nearly one hundred DUBs were identified in humans and were classed in five families (27). Each family has a specific Ub protease domain but little is known about the physiological substrates of individual members of each family. Mutations in genes expressing DUBs have been implicated in a number of diseases, including hereditary cancers and neurodegeneration (28, 29).

The UPS degrades a very large variety of proteins and many studies have described how specific substrates are recognized by the Ub system and what are the specific conditions required for the degradation process to take place. Most short-lived proteins are distinguished by localized structure determinants that target them to the ubiquitylation machinery, or sometimes directly to the proteasome. These determinants, which are named “degrons”, can serve as a degradation signal. They are defined as a minimal element within a protein that is sufficient for recognition and degradation by a proteolytic apparatus (30). They comprise primary (substrate-inherent, structural) motifs and secondary (post-translational) modifications. Among degradation signals in primary structure, the best characterized are the N-end rule system, in which the ubiquitylation of a protein is determined by the nature of its N-terminal amino acid residue; reviewed in (31), and the destruction box, which is a partially conserved 9-aa motif found in the sequence of mitotic cyclins and other cell cycle regulators (32). An important secondary modification for targeting proteins to the UPS is phosphorylation. This is the case for example of mammalian

cyclins E and D1 (33, 34), and the I κ B α inhibitor of NF- κ B transcriptional factor (35), in which specific phosphorylations serve as a recognition signal for specific Ub-protein ligases.

2. The 26S proteasome

The 26S proteasome is the key molecular complex for non-lysosomal proteolysis in eukaryotic cells and constitutes up to 1% of the total cellular protein. It is made of two subcomplexes: a central catalytic particle (CP), named 20S proteasome, associated with one or two 19S regulatory particles (RP), also called PA700. The 20S CP has a cylindrical structure in which the proteolytic active sites are compartmentalized into a catalytic chamber. Substrate access to the catalytic chamber is blocked at both ends of the CP, hence preventing an uncontrolled degradation of intracellular proteins. The binding of RP at one or both ends of the CP regulates the entry of selected substrates by opening a channel in which unfolded protein substrates are translocated to be further degraded. In addition to opening the 20S proteasome, the 19S RP is responsible for the recognition of ubiquitylated substrates, their unfolding and deubiquitylation, followed by their translocation into the 20S particle.

The 26S proteasome was discovered following the identification of a non-lysosomal proteolytic system in reticulocytes (8). The 26S proteasome was first partially purified and characterized by Hough and colleagues in 1986 as a “high-molecular-mass alkaline protease” that degrades Ub conjugates of lysozyme in an ATP-dependent manner (36) and one year later, by Waxman and colleagues, as an unusually large enzyme that is unlike any other known protease (37). A smaller multisubunit 20S protease complex was then discovered together with the larger 26S complex (38). This 20S protease was found to be ATP-independent and presented several distinct catalytic activities. Moreover, it was similar to a ‘multicatalytic proteinase complex’ that was described earlier in the bovine pituitary gland (39). Two studies further demonstrated that the 20S complex was the catalytic core of the larger 26S complex (40, 41). Direct evidence that the 26S complex results from the assembly of two distinct subcomplexes, the 20S multicatalytic particle and a 19S subcomplex, was further provided by mixing the two purified particles to generate the active 26S enzyme (42). At that time, it was already thought that the 19S subcomplex had a regulatory role.

2.1. The 19S regulatory particle

The enzymatically active 26S proteasome is generally capped at one or both ends of the central 20S catalytic core by 19S regulatory particles (RP) (**Figure 2**). The roles of the RP are to recognize ubiquitylated proteins, remove the Ub chains, unfold the substrates, and transfer them into the 20S proteasome by opening the channel conducting to the catalytic chamber.

The RP contains at least 19 distinct subunits that are subclassified into two groups: regulatory particle of AAA (ATPases Associated with various cellular Activities) ATPase subunits, called Rpt subunits, and regulatory particle of non-ATPase (Rpn) subunits. The RP is divided into two subcomplexes: the lid and the base (43).

The lid subcomplex is composed of at least 9 Rpn subunits in yeasts and among them, only Rpn11 has a well described function (43, 44). This subunit is a DUB which is responsible for the recycling of Ub molecules (45). Rpn11 cleaves the polyubiquitin chain of the substrate at a proximal site and then other DUBs cleave this chain into monomeric ubiquitins. The activity of Rpn11 seems to be critical for cell viability and proteasome function (46). The poor proteasome activity observed after inactivation of Rpn11 is presumably due to an impaired substrate translocation into the 20S CP, because of residual covalently bound Ub molecules (47). The functions of the other subunits of the lid have not yet been elucidated and are the object of intensive investigations.

The base of the RP is composed of 6 ATPase subunits (Rpt1 to Rpt6) and 4 non-ATPase subunits (Rpn1, Rpn 2, Rpn 10 and Rpn13) (43, 44). The Rpt subunits form a hexameric ring involved in substrate unfolding and translocation into the CP. Some of these subunits are also critical for the attachment of the RP to the CP and mediate the opening of the CP gate upon ATP binding (48, 49). Another essential function of the base subcomplex is the recognition of ubiquitylated substrates. This function is mediated by Rpn10 and Rpn13 subunits, which are considered as integral ubiquitin receptors (50, 51). Concerning Rpn1 and Rpn2, a structural and biochemical study showed that these two subunits form a complex which is associated with the Rpt ring and interacts with the 20S CP (52). The authors proposed that the Rpn1/Rpn2 complex participates, together with the Rpt subunits, to substrate translocation and the gating of the proteolytic channel. However, these results are in contradiction with a recent study showing, by electron microscopy, that Rpn1 and Rpn2 are not localized in the center of the ATPases (53). Another function of Rpn1 and Rpn2 would be to serve as molecular scaffolds that mediate proteasome binding of a variety of factors, including additional proteasome subunits and proteasome-associated proteins (54, 55).

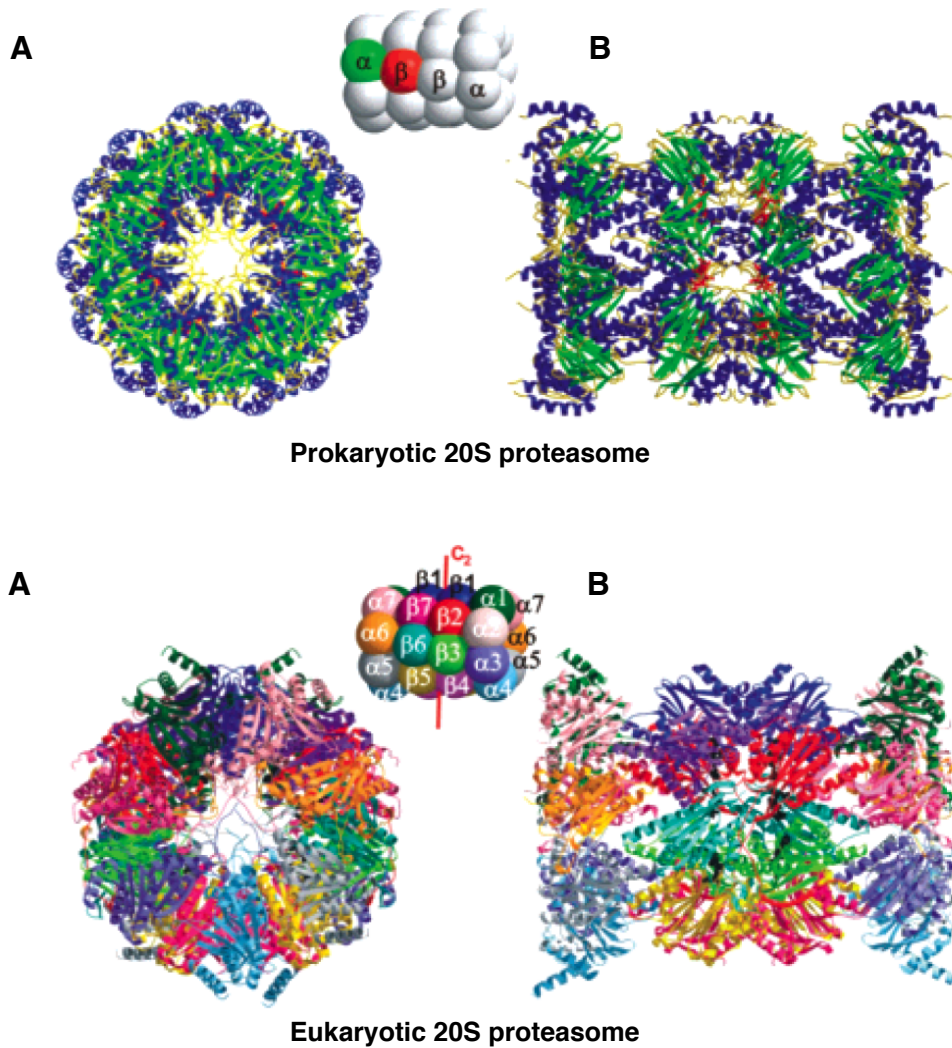
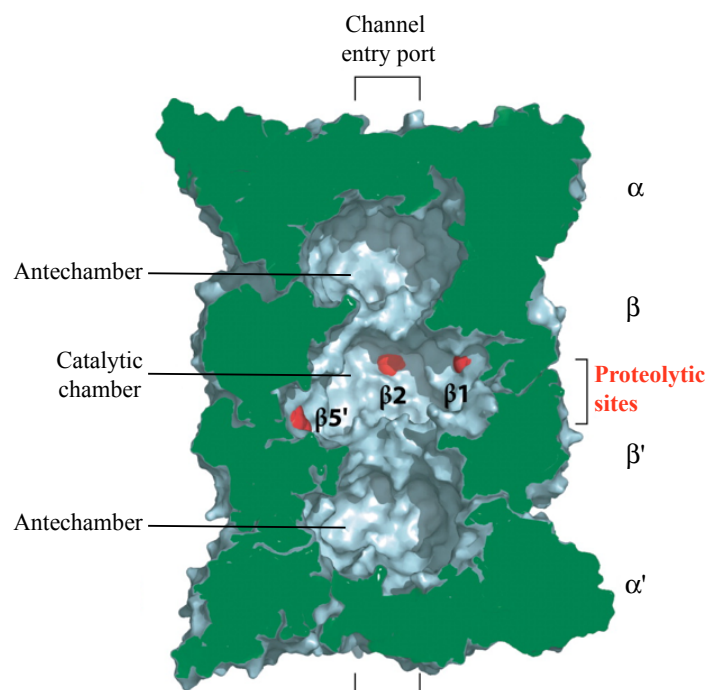


Figure 3. Structure of *Thermoplasma* (top) and yeast (bottom) 20S proteasomes in ribbon representation. (A) Front views. (B) Side views. Prokaryotic proteasomes possess one kind of α - and β -subunits, whereas eukaryotic proteasomes exhibit 7 different α - and β -subunits (82).

Figure 4. Surface representation of the eukaryotic 20S proteasome in a closed-channel conformation, showing the sequestration of the proteolytic sites (red). A median section is shown, with the slice surface in green. A bracket indicates the approximate position of the channel entry port as seen in the open state (26).



2.2. The 20S proteasome

The catalytic particle (CP) of the proteasome (namely 20S proteasome) is a well-organised protein complex of 700 kDa, inside which protein substrates are degraded. 20S proteasomes have been found in all eukaryotes (from yeast to human), in archaeobacteria and in a few eubacteria with a highly conserved structure (56). The 20S CP is made of 28 subunits that are subdivided into α - and β -subunits and are arranged in four heptameric stacked rings: two outer α -rings and two inner β -rings (57, 58). The α -subunits are inactive whereas the β -subunits form the hydrolytic chamber of the proteasome. Prokaryotic proteasomes (i.e. *Thermoplasma* proteasome) generally possess only one kind of α - and β -subunits, whereas all eukaryotic proteasomes exhibit 7 different α - and β -subunits (**Figure 3**). However, during evolution, the number of proteolytically active β -subunits has been reduced. All β -subunits of *Thermoplasma* proteasomes are active ($2 \times 7 = 14$ active sites) whereas only 3 different active β -subunits ($\beta 1$, $\beta 2$ and $\beta 5$) exist in eukaryotic proteasomes ($2 \times 3 = 6$ active sites).

In eukaryotes, the stacked rings form a $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ structure along the vertical axis, giving to the 20S proteasome a cylindrical architecture in which the 6 proteolytically active sites are sequestered (**Figure 4**). These active sites are grouped in a cavity formed by the two β -rings at the centre of the CP. This compartmentalization of the proteolytically active sites serves to protect cellular proteins from unspecific destruction. On either side of the central cavity, which is also called the catalytic chamber, a second cavity is formed between a α - and a β -ring. One of the potential functions of these two antechambers is to maintain substrates in an unfolded state (59). Unfolded protein substrates gain access into the proteasome through a narrow pore of 13 Å present in the centre of the outer α -rings, at either end of the “cylinder” (58, 60). In the latent form of the 20S CP, these pores are closed by the N termini of the α -subunits and a mechanism of gate opening is required to facilitate substrate entry. This function is carried out by some ATPase subunits of the 19S RP (49, 61). The study of the interaction between the archaeal proteasome and the PAN (proteasome-activating nucleotidases) ATPase complex, which is homologous to the ATPase ring in the base of the eukaryotic 19S RP, has provided significant insights into the mechanism of gate opening (48, 61). A conserved C-terminal hydrophobic-tyrosine-X (HbYX) motif present on PAN molecules and on two 19S ATPase subunits (Rpt2 and Rpt5) enables the binding of the RP to the CP by interacting with specific pockets formed by the α -subunits. This interaction induces a rotation in the α -subunits and stabilizes the open-gate conformation (**Figure 5**). In addition to the RP, other proteins or protein complexes, such as

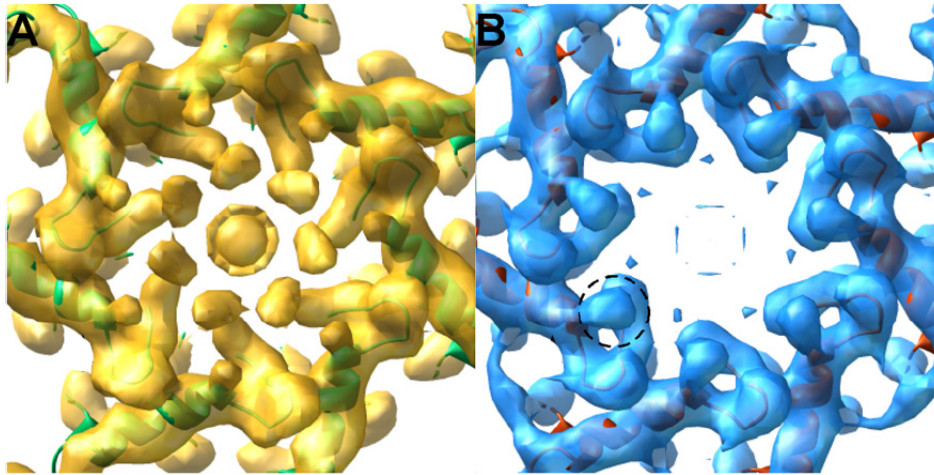


Figure 5. Density maps of the closed gate (*A*) and the open gate (*B*) of the 20S proteasome, with the structure of the docked α -subunits. The dashed circle indicates the rotation in the α -subunits induced by the binding of the 19S regulatory particle (adapted from ref. 48).

PA28 activator, can also open the CP channel and stimulate peptide hydrolysis (62) (§ I-4.2). Recently, it was demonstrated that the binding of polyUb substrates to the 19S regulator stabilizes gate opening of the 20S proteasome and, more interestingly, induces conformational changes of the 20S proteasome that facilitate channeling of substrates and their access to active sites, suggesting that polyUb substrates are able to allosterically stimulate their own degradation (63).

2.3. Peptidase activities and catalytic mechanism

Peptide bond cleavage of protein substrates occurs within the central catalytic chamber of the 20S proteasome and gives rise to a set of peptides of different lengths. In eukaryotic proteasomes, the peptidase activities are mediated by three of the seven β -subunits: **$\beta 1$** , **$\beta 2$** and **$\beta 5$** (58, 64). Mutational and structural analysis identified the N-terminal threonine (Thr1) residue of these β -subunits as the central amino acid necessary for peptide bond hydrolysis (57, 65). This catalytically active threonine is liberated during the last step of proteasome assembly by intramolecular autolysis of the prosegment present on β -subunit precursors (66, 67). The 20S proteasome is therefore classified as a threonine protease, the only one of its kind in eukaryotes. The other four β -subunits lack this N-terminal residue and are proteolytically inactive. However, they significantly contribute to the proteolytic process being directly involved in the formation of the substrate binding channels (58).

Characterization of proteasomal cleavage specificities was determined by the use of artificial tri- or tetrapeptides containing a fluorogenic leaving group at their C terminus. Irreversible proteasome inhibitors, such as peptide vinyl sulfones (§ I-5), were also used to this aim (68). Three major distinct peptidase activities were distinguished according to the nature of the amino acid residue preferred in the P1 position¹. These activities cleave synthetic peptide substrates after large hydrophobic, basic or acidic amino acid residues. They were designated, respectively, chymotrypsin-like, trypsin-like and peptidyl-glutamyl peptide hydrolysing (PGPH) activities (70). The latter is also called caspase-like or post-acidic activity. Each activity is associated with one type of catalytic β -subunit: the **caspase-like** activity is attributed to **$\beta 1$** , the **trypsin-like** activity to **$\beta 2$** and the **chymotrypsin-like** activity to **$\beta 5$** subunit (64). Two other proteolytic

¹ The positions relative to a cleavage site ($\downarrow$) are designated as described by Schechter and Berger (69): Pn-Pn-1-...-P2-P1 $\downarrow$ P1'-P2'-...-Pn-1'+Pn'.

activities were also found to contribute to peptide bond cleavage (71). A “branched-chain amino acid preferring” (BrAAP) activity, cleaving mainly after Leu residues, seems to be mediated by $\beta 1$ and $\beta 5$, and a “small neutral amino acid preferring” (SNAAP) activity, cleaving mainly after Gly, Ala and Pro residues, was assigned to $\beta 5$ (72-75). Studies using fluorogenic substrates or peptide inhibitors demonstrated that proteasomes do not select cleavage sites on a protein substrate solely according to the nature of the P1 amino acid. Indeed, substrate residues in other non-primed positions (P2, P3, ...) also influence cleavage site selection (76, 77). Since all active β -subunits carry an identical Thr proteolytic residue, their distinct preferences of cleavage are determined by the composition of the substrate binding pockets surrounding the active centres (58). The most proximal S1 and S1' binding pockets, interacting with residues P1 and P1' in the substrate, are mainly formed by residues of the active β -subunits, whereas inactive β -subunits mainly contribute to the formation of the remaining specificity pockets (78). Thus, the use of short fluorogenic peptide substrates or peptide inhibitors containing only P-residues is not sufficient to determine exactly the cleavage specificities of the proteasome and only partially reflects the physiological reality. Indeed, P' substrate residues, situated at the C-terminal side of the cleavage site and inserted in their corresponding S' binding pockets, have a significant importance on cleavage site selection (79). Therefore, substrate specificity of the proteasomal subunits reflects more physical constraints due to the slightly altered local structure around each active site, than a strict selectivity for the P1-residues of the substrate (80). Experiments using long natural peptide substrates (20-25 aa) confirmed the previous model obtained with short fluorogenic substrates but also demonstrated that some active sites can have overlapping specificities, being able to cleave notably after some hydrophobic amino acids (73). On the contrary, some other cleavages are exclusively performed by one unique active site. The degradation of a large and unmodified protein (enolase-1) using wild-type and mutant 20S proteasomes enabled to verify the reported P1-preferences of the active sites and revealed that a stretch of about 10 amino acids (P5 to P5') seems to be critical for the selection of a cleavage site (81). It was shown that proteasomes are able to cleave after almost every amino acid, but some P1 residues are disfavored (such as Pro, Gln, Asn, Gly, Thr, Ser and Lys) whereas other residues are favored (Ile, Leu, Trp, Val, Tyr, Asp and Arg). Concerning the residues located in other positions, a positive contribution to cleavage selection was also observed for Pro in P4, Gln in P5 and small residues in P1' position. The importance of the P4-residue was demonstrated earlier using peptide vinyl sulfones inhibitors (76). It should be noted that all these degradation

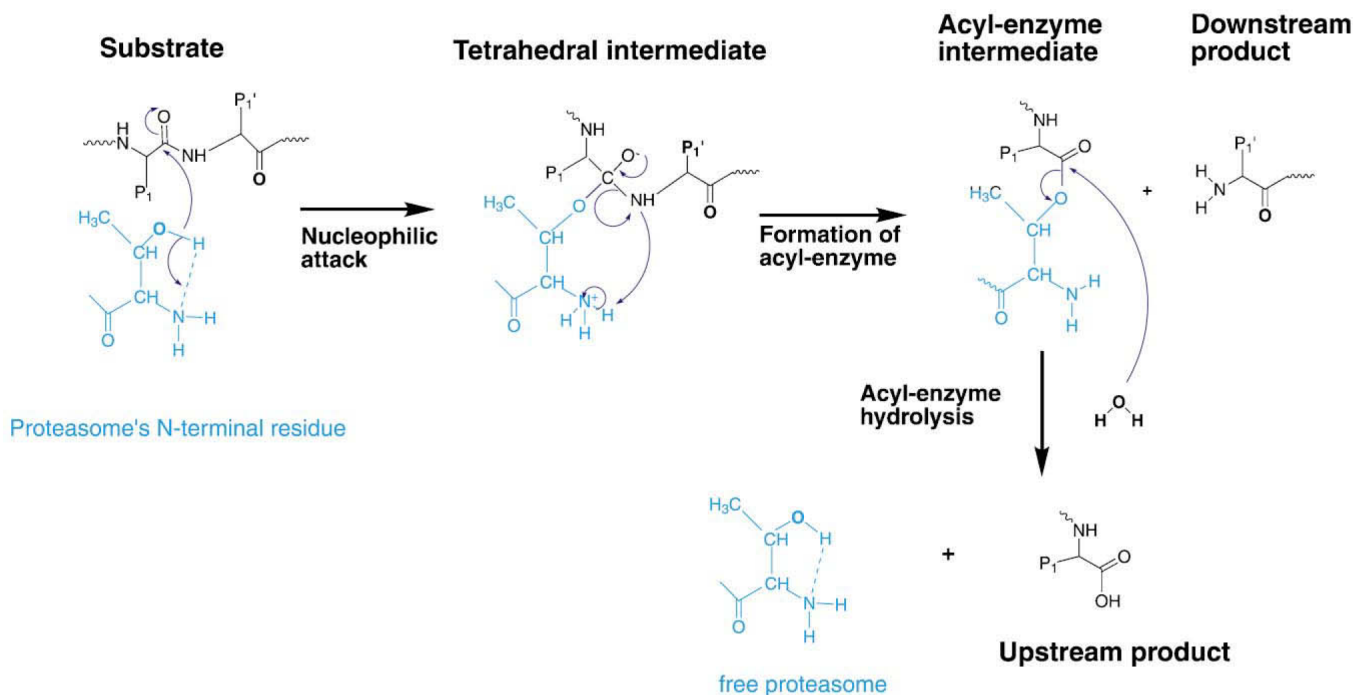


Figure 6. Catalytic mechanism of the proteasome. Substrate is in black and proteasome is in blue. The hydroxyl group of the catalytic Thr is activated by the N-terminal amino group of the same residue, so that the Thr1N represents the proton acceptor. This activation triggers the nucleophilic attack of a peptide bond, leading in a first time to the formation of a tetrahedral transition state intermediate. Molecular rearrangement of this intermediate rapidly leads to the liberation of the C-terminal downstream product, with the concomitant formation of an acyl-enzyme intermediate between the N-terminal peptide fragment and the proteasome, via an ester bond. The acyl-enzyme intermediate is further hydrolysed by a nucleophilic water molecule present at the active site, releasing the N-terminal upstream product and regenerating the active β -subunit of the proteasome (85).

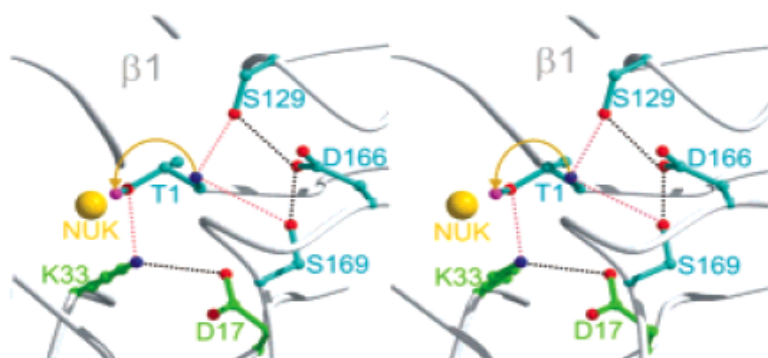


Figure 7. Stereo representation of the surroundings of the catalytic residue Thr1 in subunit $\beta 1$ of yeast proteasome. The protein backbone is drawn as white coils, whereas residues which form the active site (Thr1, Asp17, Lys33, Ser129, Asp166 and Ser169) are shown as balls-and-sticks. Lys33 forms a salt bridge with Asp17 (both residues are in green) and therefore is presumably positively charged, lowering the pKa of the ThrO γ electrostatic potential. Close to Thr1 are residues Ser129, Asp166 and Ser169 (in blue), which are required for the conformational stability of Thr1. A cluster of solvent water molecules (NUK; in yellow) is localized in the electron density close to ThrO γ and N and presumably plays a major role in the proton transfer (yellow circle) (82).

experiments were conducted in vitro with purified 20S proteasomes and may not reproduce faithfully what happens in living cells, which mainly contain 26S proteasomes.

Although the proteasome can cleave after almost every amino acid, all substrates show a unique and reproducible proteasomal product cleavage patterns. This fact is due to the limited residence time of the substrate within the proteasomal central cavity. Residues of the primed and non-primed P-sites which possess highest affinity for the distinct specificity pockets of the various substrate binding channels will have higher probability to be stabilized at the active sites to allow peptide bond cleavage (82).

It was proposed that certain active sites allosterically regulate each other's activities (83). In this model, substrates of a chymotrypsin-like site stimulate dramatically the caspase-like activity and also activate the other chymotrypsin-like site, whereas substrates of the caspase-like sites inhibit allosterically the chymotrypsin-like activity. However, this model was not supported by another study in which the inactivation of single active sites by mutagenesis does not affect the remaining active centres (74). It was further shown, using a selective inhibitor of the caspase activity, that the chymotrypsin-like and caspase sites function independently (84). Thus, it was proposed that a substrate of one subunit may regulate the activity of another via binding to non-catalytic sites rather than through binding to an active site.

The 20S proteasome is considered as a N-terminal nucleophile (Ntn) hydrolase because the N-terminal Thr of the catalytic β -subunits is responsible, through its hydroxyl group, for the nucleophilic attack of peptide bonds (65). The catalytic mechanism of the reaction is represented in **figure 6** (85) and was first proposed with the structural analysis of the proteasome from *Thermoplasma* (57). The hydroxyl group of the catalytic Thr is activated by the N-terminal amino group of the same residue, so that Thr1N represents the proton acceptor. This activation triggers the nucleophilic attack of a peptide bond leading, at first, to the formation of a tetrahedral transition state intermediate. Molecular rearrangement of this intermediate rapidly leads to the liberation of the C-terminal downstream product, with the concomitant formation of an acyl-enzyme intermediate between the N-terminal peptide fragment and the proteasome, via an ester bond. The acyl-enzyme intermediate is further hydrolysed by a nucleophilic water molecule present at the active site, liberating the N-terminal upstream product and regenerating the active β -subunit of the proteasome. Additional residues located close to the active site threonine are required for the structural integrity of the proteolytic centre and are probably involved in catalysis (**Figure 7**). These residues are invariant in the active β -subunits of the

Thermoplasma proteasome. It was proposed that, together with Thr1N and Thr1O^γ (the oxygen atom of the Thr side chain), Glu17 (Asp in yeast proteasome) and Lys33 form a catalytic tetrad (57). Lys33 is hydrogen-bonded to the active Thr1O^γ and contributes to lower its pK_a, thus enhancing its nucleophilicity. The crystal structure of the yeast 20S proteasome confirmed the importance of a cluster of water molecules (termed nucleophilic water molecules), usually located in proximity to Thr1O^γ, Thr1N, Ser129O^γ and Gly47N in the active site of β5 subunit (58). The functions of activated water molecules is to serve as the proton shuttle between Thr1O^γ and Thr1N during substrate binding, and to participate in the cleavage of the acyl-enzyme intermediate. The tetrahedral transition-state intermediate is thought to be stabilized by the NH group of Gly47, which forms the oxyanion hole by donating a proton to the negatively charged carbonyl oxygen atom of the intermediate (57).

3. Degradation of proteins by the proteasome

In vivo, the rate-limiting steps in protein degradation by the 26S proteasome are substrate binding to the 19S RP, its unfolding and translocation into the 20S CP (21). The proteasome can initiate the degradation of a protein substrate either from its N or C terminus, mostly depending on the site of ubiquitylation (86). Concerning the contribution of the different active sites to protein degradation, it was first assumed that the chymotrypsin-like sites were rate-limiting in protein breakdown by proteasomes, suggesting that the trypsin-like and the caspase-like sites play a minor role (64, 87). However, these findings observed with mutant yeast proteasomes on a few model substrates were not verified on mammalian proteasomes using active site-specific proteasome inhibitors (88). Indeed, significant inhibition of protein breakdown was achieved only when the chymotrypsin-like sites and either the trypsin-like or caspase-like sites were also inhibited. It was also observed that the relative contribution of the three active sites depended on the protein being degraded.

The protein substrates appeared to be degraded by proteasomes in a highly processive manner without the release of polypeptide intermediates, so that the rate of disappearance of protein substrates paralleled the rate of appearance of small peptidic products (89, 90). This processivity would be an inherent feature of the 20S proteasome and could be due to its structural conformation, which may contribute to delay the release of the proteolytic intermediates that are further cleaved before being released from the enzyme. However, this model remains controversial since non-processive degradation of a protein substrate was reported in one study

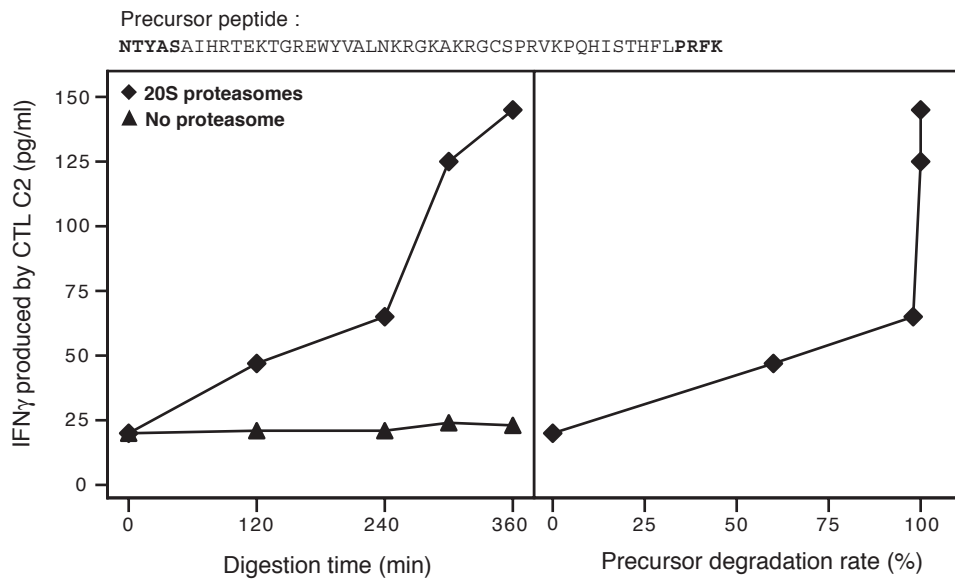


Figure 8. In vitro digestion of the indicated 49-mer precursor peptide by purified 20S proteasomes. The left panel displays the CTL recognition of digests obtained after the indicated incubation times. The right panel shows the same results in relation to the turnover of the precursor peptide, which was determined by MS. A biphasic relationship is observed between the production of the antigenic peptide and the degradation of its precursor, with a first linear phase corresponding to direct production of the antigenic peptide from the long precursor, and a second phase during which the production of the antigenic peptide keeps increasing whereas all the precursor is consumed. The latter phase seems to result from re-entry and further processing of degradation intermediates by proteasomes (93).

(91). Indeed, large degradation intermediates, which were later cleaved into small peptides, could be detected in early digests. Detection of degradation intermediates was also reported in another in vitro study using purified 20S proteasomes and polypeptide substrates (92), as well as in our hands (93). During in vitro digestions with 20S proteasomes, we observed a biphasic relationship between the production of an antigenic peptide and the degradation of its peptide precursor, with a first linear phase corresponding to direct production of the antigenic peptide from the long precursor, and a second phase during which the production of the antigenic peptide keeps increasing whereas all the precursor is consumed (**Figure 8**). The latter phase seems to result from re-entry and further processing of degradation intermediates by proteasomes. This phenomenon may be favored by in vitro digestion conditions and may not reflect what happens physiologically in cells.

Proteasomes do not degrade peptides or proteins into single amino acids, but into fragments of about 3 to 30 residues in length. The existence of a “molecular ruler” was first suggested by studying the degradation products of *Thermoplasma* proteasomes, for which about 50% of the total fragments ranged between 7-9 aa (94). In this model, the fragment length was thought to be determined by the distance between two active sites, which is 28 Å in the *Thermoplasma* proteasome, corresponding exactly to a stretch of 7-8 aa in extended conformation (57). Therefore, the reduction of the number of active sites, as found in eukaryotic proteasomes (58), predicts changing the average length of the generated fragments. However, degradation of protein substrates by yeast and mammalian proteasomes gave a similar fragment length distribution and a similar average length as found with archaeal proteasomes (81, 90), which is not therefore consistent with the “molecular ruler” model. The use of yeast proteasome mutants with reduced numbers of active sites (four or two instead of six), and the use of active site-specific proteasome inhibitors, also confirmed that the distance between these active sites does not influence the size distribution of the fragments produced (81, 90). Both 20S and 26S proteasomes are able to degrade in vitro denatured or unfolded proteins without prior ubiquitylation. Two studies compared protein degradation by both proteasome complexes and showed that, even though the overall size distribution of the peptide products was similar, 26S proteasomes produce slightly shorter fragments than 20S proteasomes (90, 95). However, precautions must be taken with these results because peptides with less than 5 aa could not be detected in these studies. The pool of generated peptides was only partially identical (about 25%) between the two proteasomes species, suggesting that 20S and 26S proteasomes, despite their identical composition in proteolytic active sites, could cleave proteins at different sites. The authors speculated that fixation of the 19S RP could bring about conformational changes in the

quaternary structure of the 20S CP, potentially inducing a change in cleavage specificity (95). However, no structural analysis supporting this hypothesis is available to date.

Proteins are not always entirely degraded into small peptides by proteasomes. Some examples of partial proteolysis were reported and the most studied was activation of the NF- κ B transcription factor (14). Partial proteasomal processing was also described for the two yeast NF- κ B-related transcription factors, SPT23 and MGA2, which are only released after the partial degradation of their ER membrane-bound precursors (96). NF- κ B can only be activated after the partial processing of p105 to p50. It was proposed that the generation of p50 occurs cotranslationally by a proteasome-mediated process that ensures the production of both p50 and p105 (97). The cotranslational folding of the N-terminal part of p105 (corresponding to p50) may protect this region from proteasomal degradation, whereas the C-terminal part, which remains attached to the ribosome and which is not tightly folded, can be degraded by the proteasome. This model proposes that the random pausing of the ribosome after synthesizing approximately 530 residues leads to the degradation of the C-terminal part and the generation of p50. However, when the ribosome does not pause, a complete p105 molecule is synthesized, which cannot undergo further processing. The N- and C-terminal domains are separated by a 23-aa glycine-rich region which is essential for p50 processing (98). The proposed model of cotranslational processing of p50 suggests that the glycine-rich region may form a loop that can be inserted into the proteasome, allowing processive degradation until the tightly folded N-terminal domain is reached (97). This model implies therefore that the degradation of the substrate begins at internal peptide bonds after the translocation of a polypeptide chain loop through the narrow gate of the 20S proteasome. To support this hypothesis, it was shown that the inner channel of the proteasome can accommodate more than one polypeptide chain (99). An endoproteolytic activity was further demonstrated by showing that 20S and 26S proteasomes were indeed able to degrade circular or bilaterally protected protein substrates (100). The mechanism of partial proteolysis was confirmed later for the yeast SPT23 and MGA2 proteins, for which proteasomal degradation is initiated by an internal cleavage, followed by bidirectional proteolysis of the polypeptides until tightly folded structures are reached (101). This study also pointed out that this mechanism may be relevant for the complete degradation of proteins. The model of cotranslational processing of NF- κ B was not supported by a more recent study in which translation of full length p105 is required before the proteasomal processing of p50 (102). Nevertheless, they confirmed that the glycine-rich region is essential for p50 processing by serving as a stop signal for proteasomal

degradation. They also supported that the processing of p50 is indeed initiated through an endoproteolytic cleavage event.

Many studies described the ability of the 20S proteasome to catalyse the degradation of natively disordered physiological substrates, suggesting that they possess inherent features that can promote gate opening of the α -ring of the CP. This mechanism supports therefore a potential role for the free 20S proteasomes found in the absence of 19S RP in different cell types (103). In line with this idea, many proteins were found to be degraded in cells by the 20S proteasome without prior ubiquitylation. This type of degradation is called “by default” and seems to be a wide and general phenomenon (104). For example, many oxidized, unfolded and “aged” proteins were found to be Ub-independent substrates due to the exposure of hydrophobic patches, which are suspected to stimulate recognition by proteasomes (105). However, for most substrates it is still unclear what constitutes the degradation signal by the 20S proteasome and how interaction with different proteins protects them from degradation. Remarkably, the 26S proteasome is also responsible for Ub-independent protein degradation (106).

4. Modulation of proteasome content and activity

Proteasome activity in the immune system can be regulated by the replacement of the proteolytically active subunits of the CP, resulting in the formation of slightly different particles, the immunoproteasome and the thymoproteasome, which can generate altered sets of peptide products. Proteasome activity can be also modulated by the replacement of the 19S RP by complexes known as proteasome activators. These complexes bind to the cylinder end of the CP and open its axial channel. We will focus here on the most studied proteasome activators which are PA28, also termed 11S regulator, and the large nuclear protein PA200.

4.1. The immunoproteasome

Higher eukaryotes contain two genes for each of the three catalytic β -subunits. The immunomodulatory cytokine interferon- γ (IFN γ), which is produced by some activated T lymphocytes and natural killer cells, upregulates the product of three of these genes. These IFN γ -inducible catalytic subunits are incorporated in newly synthesized proteasomes instead of their constitutive counterparts: **β 1i (LMP2)** replaces β 1, **β 2i (MECL1)** replaces β 2 and **β 5i (LMP7)**

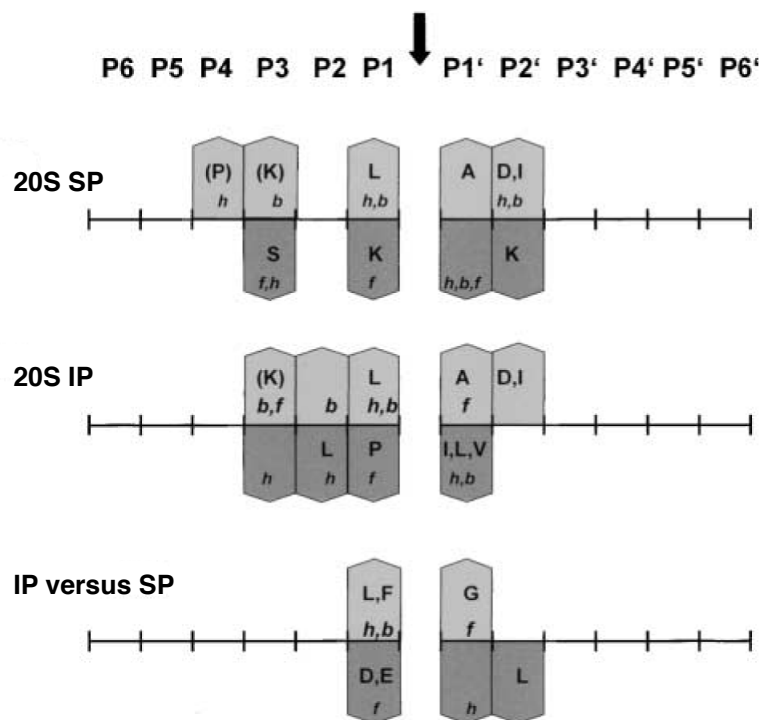


Figure 9. Summary of cleavage motifs. Amino acid preferences at positions flanking the cleavages performed by the standard proteasome (SP) or the immunoproteasome (IP) during enolase digestion are indicated by bars pointing up (enriched) or down (depleted). Amino acid frequencies around cleavage sites were compared with amino acid frequencies in enolase. IP versus SP indicates a comparison of amino acid preferences between both types of proteasome. Bars pointing up indicate a IP preference, bars pointing down indicate a SP preference. *h*, hydrophobic; *f*, flexible; *b*, bulky (122).

replaces $\beta 5$ (107, 108). Besides IFN γ , tumor necrosis factor- α (TNF α), a pleiotropic proinflammatory cytokine known to up-regulate MHC class I molecule expression, was also shown to induce the expression of at least two of these three inducible subunits, i.e. $\beta 1i$ and $\beta 5i$ (109). Apart from their induction by cytokines or infection, these subunits were also reported to be induced by damages like heat stress, arsenic trioxide or nitric oxide (110-112). Interestingly, the genes of $\beta 1i$ and $\beta 5i$ are encoded in the MHC locus on chromosome 6, together with the genes encoding MHC molecules, the transporter associated with antigen processing (TAP), and other genes related to immune response (113, 114). It was also observed that the incorporation of the inducible catalytic subunits alters the peptidase activities of the proteasome in ways that can enhance the generation of some antigenic peptides (115-117). Because of this role in antigen presentation, $\beta 1i$, $\beta 2i$ and $\beta 5i$ were named “immunosubunits” and the proteasome particles containing these subunits were named “immunoproteasomes” (IP) (107). In contrast, the constitutively expressed proteasome is called standard proteasome (SP), or constitutive proteasome. Thus, at least two types of proteasomes were thought to exist: SP that are expressed in all cells and IP that are formed upon exposure of cells to IFN γ . However, constitutive expression of IP is observed in some immuno-relevant tissues such as spleen, small intestine as well as in thymus (118, 119).

Studies using fluorogenic or naturally occurring peptides as substrates demonstrated that the incorporation of the immunosubunits has an influence on the cleavage site selection (116, 120). Post-acidic cleavages were found to be reduced whereas chymotrypsin-like, trypsin-like and BrAAP activities were enhanced for immunoproteasomes, without changing the overall rate of hydrolysis. These observations were further confirmed using full-length protein substrates (121-123). Digestion of enolase by SP and IP provided a detailed analysis of the modifications of cleavage motifs after immunosubunit incorporation (122). No difference was observed in the average length of fragments produced by either type of proteasome, as well as in the rate of protein breakdown. However, by comparing flanking residues around cleavage sites, it was shown that SP and IP display partially overlapping but different cleavage specificities (**Figure 9**). The enhanced preference of immunoproteasomes for hydrophobic residues in P1 position was confirmed, and more strikingly for the branched amino acid leucine, as well as the decreased preference for acidic residues at the same position. However, no higher trypsin-like activity was observed for immunoproteasomes when using entire proteins as substrates. In addition, an enrichment of Gly and a concomitant decrease of large hydrophobic amino acids in P1' position was found. These studies strongly suggested that the immunoproteasome reduces the production

of peptides with charged C termini (Asp, Glu), but increases the liberation of peptides with hydrophobic C termini that are more suited to bind to the TAP transporter and to MHC class I molecules. Thus, the new pattern of peptides that are produced by immunoproteasomes has been suggested to be more favorable to antigen presentation, so that immunoproteasome expression might be generally associated with a more efficient production of class I antigenic peptides (124). It was shown later, with several studies on the differential processing of antigenic peptides between SP and IP, that the situation is indeed more complicated (§ II-3.3). A more general physiological function of immunoproteasomes, apart from antigen processing, was proposed recently. Upon IFN γ -induced oxidative stress, the concomitant induction of immunoproteasomes is required to efficiently degrade the enhanced pool of polyUb-substrates and preserve cell viability (125). So, rather than having a specific role in the production of antigenic peptides, the authors proposed that immunoproteasomes increase the peptide supply for antigen presentation as part of a more general role in the maintenance of protein homeostasis.

No crystallographic structural analysis of the immunoproteasome complex is available yet. However, the superposition of the amino acid sequence of constitutive and immuno-catalytic subunits and modelling experiments revealed high conservation of the substrate binding pocket residues (58, 79). This is particularly true for $\beta 2$ and $\beta 5$, compared to $\beta 2i$ and $\beta 5i$ subunits. Therefore, the enhanced preference of immunoproteasomes for branched and hydrophobic amino acids at P1 position may not result from exchange of $\beta 5$, which mediates the chymotrypsin-like activity, for $\beta 5i$ subunit. However, the replacement of $\beta 1$ for $\beta 1i$ is predicted to cause the formation of a smaller and more apolar S1 pocket (58, 126). The exchange of $\beta 1$ for $\beta 1i$ would be therefore responsible for the reduced activity towards acidic residues and the higher BrAAP and chymotrypsin-like activities of the immunoproteasome. According to these modelling experiments, the major changes between constitutive and immunosubunits seem to affect the primed substrate-binding channels, which accommodate the peptide sequence at the C-terminal side of the cleavage site (79). This would contribute to explain the significant effect of immunosubunits incorporation on the substrate preference and cleavage pattern.

Incorporation of the immunosubunits is cooperative: $\beta 2i$ is incorporated only if $\beta 1i$ is present, whereas $\beta 1i$ incorporation is independent of $\beta 2i$ (127, 128). Moreover, the $\beta 5i$ subunit seems to be required for an efficient immunoproteasome maturation. This cooperative mechanism favors the assembly of homogeneous immunoproteasomes, containing all three inducible subunits. However, the existence of 20S complexes in which immunosubunits and constitutive subunits

are present together was strongly suggested by chromatographic and enzymological analyses (129, 130). These proteasomes, which are intermediate between the standard proteasome and the immunoproteasome were identified and further characterized in our laboratory using a series of novel antibodies recognizing catalytic subunits of the human proteasome in their native conformation (131). Two intermediate proteasomes were identified : one containing only $\beta 5i$ and the other containing $\beta 1i$ and $\beta 5i$. These intermediate proteasomes represent 30 to 50% of the proteasome content of human liver, colon, small intestine and kidney. They are also present in tumor cells and dendritic cells. Since intermediate proteasomes appear to present activity profiles that are different from the SP and the IP, modulation of proteasome content may affect proteasome functions, such as antigen processing, activation of transcription factors, as well as regulation of the cell cycle.

4.2. PA28 activator

In higher eukaryotes, two other genes are found in the MHC locus of chromosome 6 adjacent to the inducible catalytic subunits of the proteasome. These genes code for two homologous subunits of 28 kDa, PA28 α and PA28 β , which form an additional proteasome-regulatory complex, termed PA28 activator or 11S regulator. PA28 α and PA28 β , which are abundant in vertebrate immune tissues, are mostly located in the cytoplasm. Synthesis of PA28 α and β subunits is stimulated by IFN γ , leading to the prevalent hypothesis that PA28 activator plays an important role in the immune response. However, PA28 is expressed at a basal level in all cell types of higher eukaryotes. PA28 was first identified and characterized as a ring-shaped multimeric complex of ~180 kDa that can bind to one or two ends of the 20S proteasome, thereby stimulating its capacity to hydrolyse small peptides but not intact or polyUb proteins (132, 133). In vivo, this complex has been reported to be hexameric (3 α 3 β) or heptameric (3 α 4 β) (134, 135). PA28 does not seem to be important in protein degradation since the formation and function of PA28-proteasome complexes do not require ATP, unlike the cellular breakdown of proteins. Moreover, as PA28-proteasomes fail to degrade large protein substrates with native or denatured structures that are ubiquitylated or not, these complexes did not appear to play a central role in initiating the cleavage of protein substrates. Rather, they presumably have a stimulatory effect on the degradation of polypeptides of intermediate size that are generated by 26S proteasomes, implying that the 26S proteasome and the PA28-proteasome may function sequentially on the same substrate molecules.

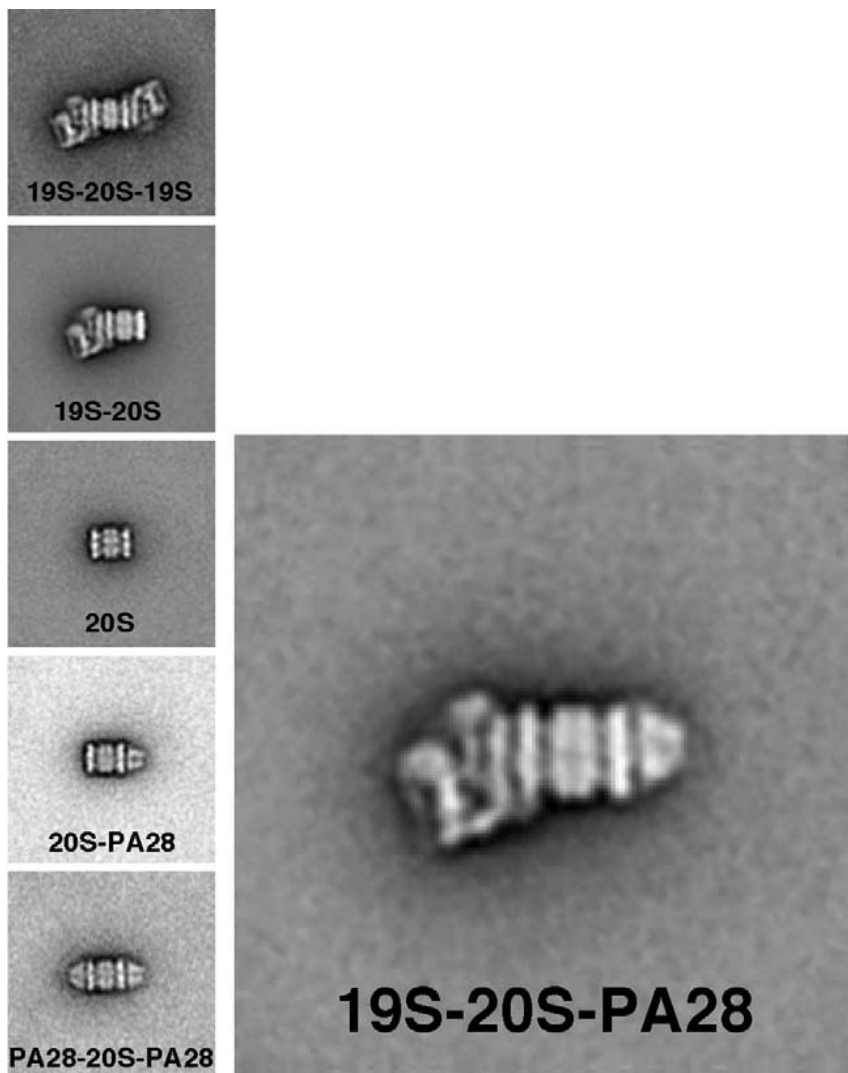


Figure 10. Electron micrographs of a negatively stained proteasome mixture showing the six different types of proteasome complexes (144).

The co-crystal structure of the trypanosomal homologue of PA28 (PA26) together with the 20S CP shows a conformational change in the N-termini of proteasomal α -subunits that leads to the opening of the gate in the α -ring (62, 136). However, the mechanism of gate opening mediated by PA28 (PA26) seems to be slightly different from that of the 19S regulatory particle (48). The ability of PA28 to open the gate of the 20S proteasome would be responsible for the enhancement of intrinsic proteasome activity, although this mechanism is not yet fully understood. A large variety of biochemical actions have been proposed for the PA28 activator. Allosteric activation of the proteasomal active sites upon PA28 binding, concomitant with a stimulation of peptide entry into the 20S CP (132), and a stimulation of peptide exit were first reported (62). PA28 was also predicted to facilitate the binding of proteasomes to chaperones or to components of the ER membrane, suggesting a potential role in MHC class I antigen presentation (137). Furthermore, in vitro, the presence of PA28 enhanced the production, by proteasomes, of peptide fragments generated by two flanking cleavages while the generation of single-cleavage products was strongly reduced (138, 139). This increase in coordinated double cleavages by PA28-proteasomes was directly associated with the enhanced production of some MHC class I ligands (138, 140). Several other reports demonstrated that the expression of PA28 correlated with an increase in processing of specific MHC class I epitopes, probably by enhancing the cleavage strength of minor cleavage sites; reviewed in (141). These data, and also a mutational analysis of PA28, suggested that the binding of this proteasome activator may induce conformational changes within the 20S CP, modifying the accessibility of the active sites (142).

Because 20S proteasomes contain two identical outer α -rings, a given 20S complex can accept two different regulators on opposite sides, leading to many potential distinct proteasome complexes. Six native proteasome populations were identified in intact cytosol of mammalian cells using two-dimensional non-denaturing electrophoresis and tandem mass spectrometry (143), but also using electron microscopy from in vitro purified components (144). These complexes are 20S, singly and doubly PA28-capped (PA28-20S and PA28-20S-PA28), singly and doubly 19S-capped (19S-20S, 19S-20S-19S) and **hybrid proteasomes** (PA28-20S-19S) (**Figure 10**). All cellular proteasome complexes are shown to be highly dynamic and undergo rapid exchange or redistribution of regulatory particles in response to changes in cellular environment (143). The activity of hybrid complexes PA28-20S-19S towards peptide or protein substrates was compared to the activity of 26S proteasomes (144). Hybrid proteasomes were found to enhance several fold the hydrolysis of fluorogenic peptides, but do not alter full-length

protein hydrolysis. In such asymmetric complexes, ubiquitylated and non-ubiquitylated proteins most likely initially bind to the 19S RP, since their degradation is an ATP-dependent process which occurs at similar rates as that of 26S proteasomes. Furthermore, this study showed that hybrid proteasomes generate, from protein substrates, a pattern of peptides different from those generated by 26S proteasomes, without altering the average length of the products. It remains unclear, however, how exactly the presence of PA28 alters the nature of the peptides produced, and if PA28-proteasomes and hybrid proteasomes play a specific role in mammalian cells.

A third structurally-related PA28 subunit, known as **PA28 γ** , is found in both vertebrates and invertebrates. While PA28 α and PA28 β are mainly cytosolic, PA28 γ is predominantly located in the nucleus (145). PA28 γ subunits form a heptameric ring, structurally analogous to PA28 activator. PA28 γ complex apparently has no ATPase activity to support unfolding and no Ub-recognition capacity but it is able to open the CP channel (146). PA28 γ is not regulated by IFN γ and does not seem to be involved in the processing of antigens. A study using mice lacking the PA28 γ gene indicated a potential role in the regulation of cell proliferation and body growth (147). Several reviews have discussed the functions of PA28 γ and mentioned its importance in Ub-independent nuclear proteolysis (148), as well as in apoptosis (149).

4.3. PA200 activator

The proteasome activator PA200 has been identified as a 200-kDa protein bound to nuclear 20S proteasomes and has been isolated from bovine testis (150). By interacting with the α -subunits, PA200 opens the axial channel of 20S CP (151). Analogous to the PA28 activator, PA200 was found to stimulate the hydrolysis of small fluorogenic peptides, most notably after acidic residues, but not of folded proteins (150). The existence of hybrid proteasomes consisting of 19S-20S-PA200 was also reported in yeast and mammals (150, 152). The expression of PA200 is induced by ionizing radiation and accumulates on chromatin, suggesting a potential function in DNA repair (150, 153). It was further shown that hybrid proteasomes 19S-20S-PA200 are indeed required, with their enhanced post-acidic activity, for the cellular response to ionizing radiation and maintenance of genomic integrity (154).

4.4. The thymoproteasome

A novel catalytic β -subunit exclusively expressed in the thymus was discovered a few years ago in human and mouse (155). This subunit is structurally related to $\beta 5$ and $\beta 5i$ subunits, and was named $\beta 5t$, where 't' stands for 'thymus'. $\beta 5t$ is incorporated into proteasomes, instead of $\beta 5$ or $\beta 5i$, together with $\beta 1i$ and $\beta 2i$ subunits, forming an alternative proteasome type, named thymoproteasome. This novel type of proteasome is exclusively expressed in cortical thymic epithelial cells (cTEC), which are responsible for the positive selection of developing T cells in the thymus. Together with thymoproteasomes, standard and immunoproteasomes are present in cTEC, but the relative abundance of each proteasome type in these cells has not been fully elucidated. It was demonstrated, using small fluorogenic peptides as substrates, that the incorporation of $\beta 5t$ instead of $\beta 5$ caused a marked reduction in chymotrypsin-like activity, without affecting caspase- and trypsin-like activities (155). However this has been demonstrated with cells expressing $\beta 1$ and $\beta 2$, whereas $\beta 5t$ is normally associated with $\beta 1i$ and $\beta 2i$ in the thymoproteasomes of cTEC. Considering that proteasomes are essential for the production of MHC class I ligands and that $\beta 5t$ attenuates the proteasomal chymotryptic activity, it is thought that the role of thymoproteasomes in cTEC would be to produce moderate avidity MHC class I peptides rather than high-affinity peptides, which would support positive selection (148). This is consistent with a recent study of the same group, which showed that cTEC displayed thymoproteasome-specific peptide-MHC class I complexes that are essential for the positive selection of the repertoire of CD8⁺ T cells (156).

5. Proteasome inhibitors

Studies into the chemical synthesis of proteasome inhibitors were initiated during the early 1990s. They first focused on the modification of the amino acid sequence of the serine/cysteine protease inhibitors that were shown to inhibit the 20S proteasome (70). The most representative proteasome inhibitors are indicated in **figure 11** (157).

Peptide aldehydes, such as MG115, **MG132** (Z-Leu-Leu-Leu-al) or PSI, were first developed and widely used to study proteasome structure and biology, particularly by addressing its role in the degradation of most cell proteins and generation of MHC class I ligands (16). These compounds can bind to all proteasomal active β -subunits but have a significant preference for the

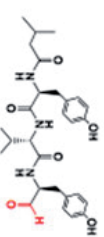
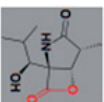
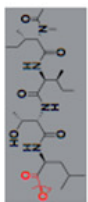
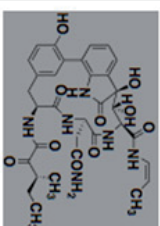
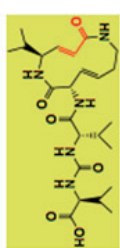
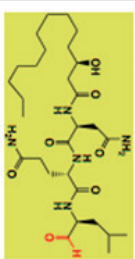
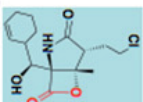
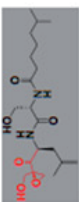
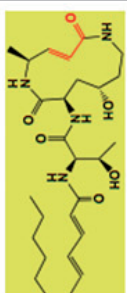
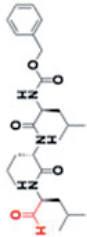
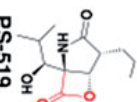
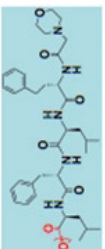
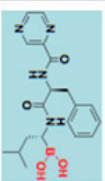
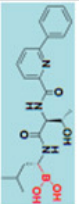
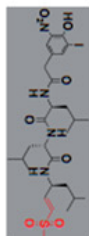
Class Origin	Aldehydes	β -lactones	Epoxyketones	Boronates	Vinyl sulfones	Cyclic peptides**	Macrocyclic vinyl ketones
Natural products	 Tyropeptin A	 Omuralide (<i>clasto</i> -lactacystin- β -lactone)	 Epoxomicin			 TMC-95A	 Syringolin A
	 Fellutamide B	 Salinosporamide A (NPI-0052)	 Eponemycin				 Glidobactin A
Synthetic	 ALLN  MG-132 PSI (Z-Ile-Glu(OtBu)-Ala-Leu-al)	 PS-519	 Carfilzomib (PR-171)	 Bortezomib  CEP-18770 MG-262 (Z-LLL-boronate)	 NLVS  ZLVS	TMC-95 analogues	

Figure 11. Major classes of proteasome inhibitors and their selected representatives.
 “Founding members” of the family are highlighted in gray. Clinically used inhibitors are highlighted in blue and novel inhibitors are in yellow (157).

$\beta 5$ subunit (58). Peptide aldehydes are reversible inhibitors that share similar peptide backbone and aldehyde functional group with known protease inhibitors such as calpain inhibitor I (ALLN). For this reason, and because the functional aldehyde group is highly reactive, all peptide aldehydes are rather unspecific to the proteasome and also block a broad range of serine and cysteine proteases (85). However, they are still widely used as tools to investigate regulation of the UPS.

Other known peptide-based protease inhibitors possessing different pharmacophores have been developed as proteasome inhibitors. These are peptide vinyl sulfones and peptide boronates.

Peptide vinyl sulfones are irreversible inhibitors of proteasome that covalently modify its catalytic β -subunits (158). They do not block serine proteases but display high specificity for intracellular cysteine proteases such as cathepsins (159). Peptide vinyl sulfones, in particular the compound Ada-(Ahx)₃-(Leu)₃-vs, generally bind to all proteolytically active β -subunits of both the standard and the immunoproteasome with approximately equal efficiency (68). Such broad-spectrum peptide vinyl sulfones were therefore labeled with either a radioisotope, a biotin moiety or a fluorescent tag, and used in the profiling of proteolytic active subunits of the proteasome in different tissues and cells (§ I-2.3). In addition, a peptide vinyl sulfone was designed to specifically bind to the caspase-like sites (160). This compound was shown to inactivate both $\beta 1$ and $\beta 1i$ subunits and can be used, for example, to study the roles of these subunits in the generation of specific antigenic peptides (161)

Peptide boronates are more potent and more selective proteasome inhibitors than peptide aldehydes and vinyl sulfones. Inhibition of few serine proteases by many peptide boronates was reported, but is about 1000-fold weaker than that for the proteasome (162). These compounds are considered as reversible inhibitors, forming a tetrahedral adduct with an active threonine, as was observed for peptide aldehydes. However, they possess much lower dissociation rates than peptide aldehydes due to the stabilization of the adduct with hydrogen bonds, so that the inhibition is practically irreversible over hours. Peptide boronate **PS-341** (pyrazylcarbonyl-Phe-Leu-boronate), later renamed **bortezomib**, is the only proteasome inhibitor used as drug therapy to date and has been approved for the treatment of multiple myeloma and mantle cell lymphoma. Aside from the proteasome, no other target of bortezomib could be identified, demonstrating the high selectivity of this compound (163). Concerning the proteasomal β -subunit binding

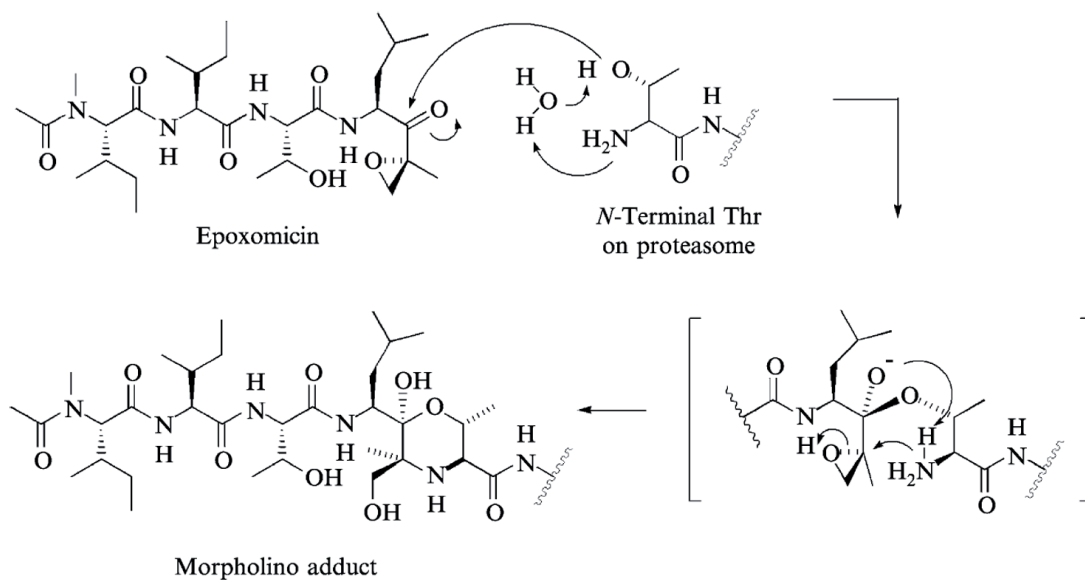


Figure 12. The mechanism of proteasome inhibition by epoxomicin is proposed on the basis of the x-ray structure of yeast 20S proteasome–epoxomicin complex. It is postulated that the unique specificity of epoxomicin is due to the formation of an unusual six-membered morpholino ring between Thr-1 of the catalytic subunit of 20S proteasome and the epoxyketone pharmacophore of epoxomicin (167).

specificity, bortezomib preferentially targets the $\beta 5$ active site, and to a lesser extent the $\beta 1$ site, while $\beta 2$ subunit does not seem to be affected (164). In addition to synthetic peptide inhibitors, different natural compounds also provide both peptide and non-peptide proteasome inhibitors.

Peptide epoxyketones, such as eponemycin and **epoxomicin**, were discovered based on their antiinflammatory and antitumor activities in mice (165, 166). Epoxomicin, which was isolated from an actinomycete, is highly specific for the proteasome and does not inhibit other classes of proteases. The high specificity of this class of inhibitors is due to their unique mechanism of interaction with the active sites of the proteasome. This mechanism was revealed by the crystallographic study of the yeast 20S proteasome complexed with epoxomicin showing the formation of an unusual covalent morpholino adduct between the epoxyketone pharmacophore and the hydroxyl and amino groups of the catalytic threonine (167) (**Figure 12**). Enzymatic assays showed that epoxomicin primarily affects the chymotrypsin-like activity, whereas tryptic-like and caspase-like activities are inactivated, respectively, at 100- and 1000-fold slower rates (168). Peptide epoxyketones with activity-specific inactivation effects were synthesized, such as the YU101, which displays specificity for the chymotrypsin-like activity (169) and the YU102, which displays specificity for the caspase-like activity (84). Another synthetic compound, derived from dihydroeponemycin, was shown to specifically inactivate the $\beta 1i$ subunit of the immunoproteasome, while epoxomicin covalently modify $\beta 5$ and $\beta 5i$, and, to a lesser extent, $\beta 2$ and $\beta 2i$ (170).

Lactacystin, which belongs to the **β -lactones**, is a non-peptidic natural compound produced by *Streptomyces*. It was shown to covalently bind mainly to the $\beta 5$ and $\beta 5i$ subunits of the proteasome, inactivating effectively and irreversibly the chymotrypsin-like activity (171). The other proteolytic sites were also modified and irreversibly inhibited, but at much slower rates (172). Lactacystin itself is not active against proteasomes and, in aqueous solution around neutral pH, is spontaneously hydrolysed in *clasto*-lactacystin- β -lactone, also termed omuralide, which acts as the functional reactive group (173). Although considered as a selective irreversible proteasome inhibitor, lactacystin was also shown to inhibit a few serine proteases, such as cathepsin A, which is a lysosomal carboxypeptidase (174), and tripeptidyl peptidase II (TPPII), a cytosolic giant protease that possesses a role of N-terminal trimming of MHC class I antigenic peptides (175) (§ II-4).

Salinosporamide A is another natural β -lactone, which was isolated from a marine actinomycete (176). It is a potent and selective inhibitor of the proteasome, currently undergoing clinical studies as potential drug for cancer treatment (177).

Homobelactosin C is derived from the natural belactosin C produced by *Streptomyces*. This product displayed high antitumor activity due to proteasome inhibition (178) and unique binding mode to the N-terminal catalytic threonine, providing information about the composition of proteasomal primed substrate-binding sites (79).

A novel family of bacterial plant pathogen products which irreversibly inhibit all three proteolytic activities of the proteasome, was recently discovered (179). These compounds are named syrbactins and include **syringolin A** and **glidobactin A**.

Beside these covalent inhibitors, various non-covalent proteasome inhibitors were also described. Examples are the natural product **TMC-95A**, isolated from the plant pathogen *Apiospora montagnei*, and its synthetic analogues (180). TMC-95 compounds, which are not related to any other proteasome inhibitors mentioned previously, consist of a heterocyclic ring system made of modified amino acids. According to a crystallographic study, TMC-95A is noncovalently linked to all three proteolytic active sites, and does not modify their N-terminal threonines (181). Instead, a tight network of hydrogen bonds connects TMC-95A to the proteasome and stabilizes its position.

A novel series of non-covalent di-peptide inhibitors of the proteasome was recently described (182). These compounds were shown to be entirely selective for the $\beta 5$ subunit, thus allowing discrimination between the standard proteasome and the immunoproteasome.

6. ER-associated degradation (ERAD) pathway

In addition to the degradation of cytosolic proteins, the UPS is responsible for the degradation of the majority of secretory and membrane proteins that fail to correctly fold in the ER. These aberrantly folded proteins are retained in this compartment and can be targeted for ER-associated degradation (ERAD) by a process known as ER quality control. In yeast, three different ERAD pathways can be distinguished according to the location of the misfolded domain on the substrate. When proteins are misfolded in the ER lumen, they are degraded through the ERAD-L pathway. When membrane proteins are misfolded in the membrane spanning domain or in the cytosolic domain, they are degraded through the ERAD-M or ERAD-C pathways, respectively (183). These three pathways possess distinct effectors that are responsible for ERAD substrate

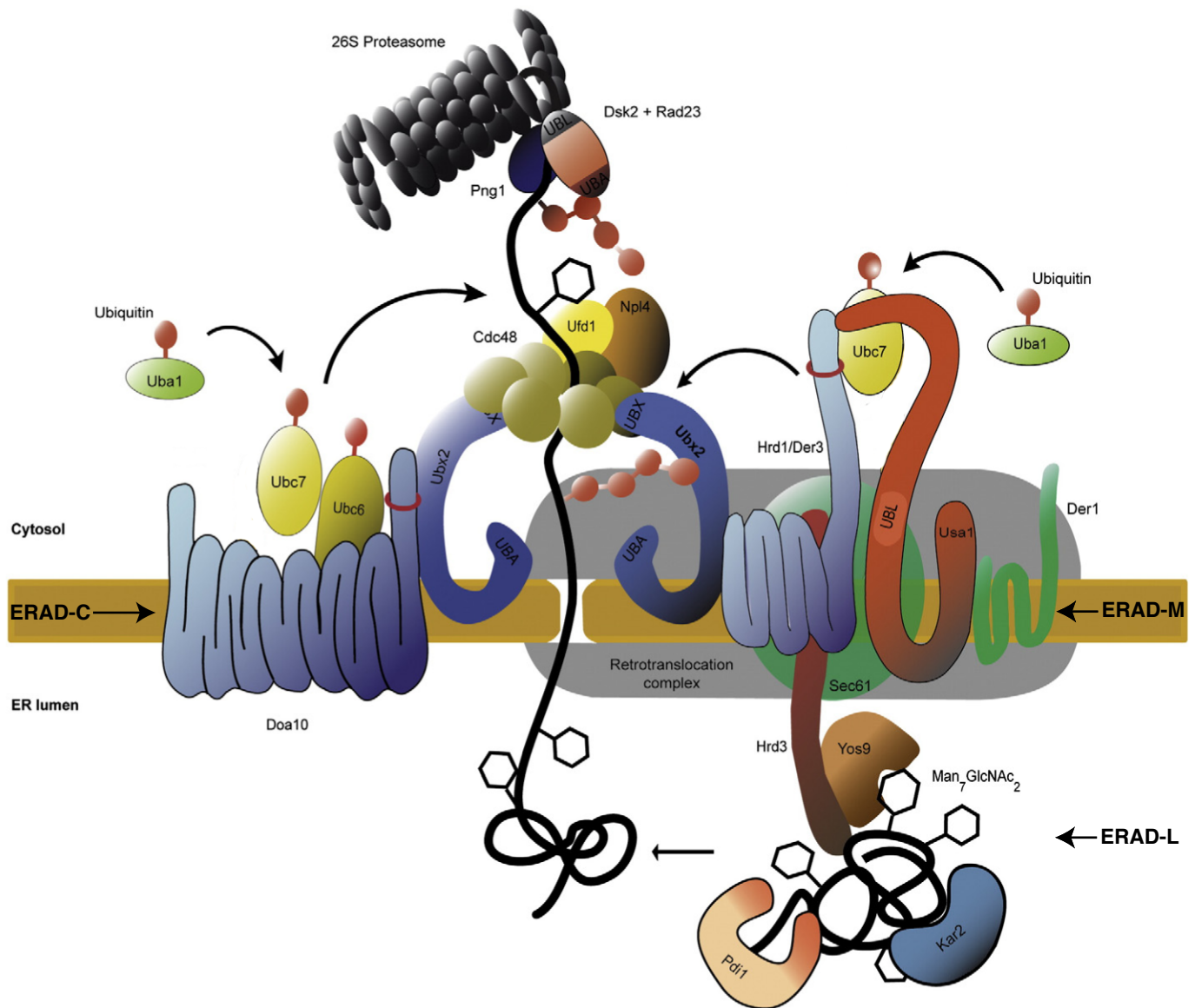


Figure 13. The **ERAD machinery** for delivery, ubiquitylation and degradation of ERAD-L, ERAD-C and ERAD-M proteins in yeast. ERAD-L and ERAD-M substrates are delivered to the ubiquitylation machinery associated with the retrotranslocation complex by ER chaperones such as Pdi1 and Kar2, and the chaperone-like lectin Yos9. The retrotranslocation complex is composed by different ER membrane proteins including Sec61, Hrd3, Usa1 and Der1, and by membrane-bound E3 ubiquitin ligase Hrd1/Der3, which is responsible for the ubiquitylation of ERAD-L and ERAD-M substrates on the cytosolic side. ERAD-C substrates are delivered to the retrotranslocation complex by cytosolic chaperones (not represented here) and are ubiquitylated by the E3 ligase Doa10. The E1 and E2 enzymes implicated in the ubiquitylation of ERAD substrates are the Ub-activating enzyme Uba1 and the Ub-conjugating enzymes Ubc6 and Ubc7. Polyubiquitylated proteins are pulled from the ER membrane by the AAA ATPase Cdc48, which is in complex with Ufd1 and Npl14 and linked to the ER membrane by Ubx2. Cdc48 complex then delivers the substrates to the proteasome via the Ub receptors Dsk2 and Rad23. The glycanase Png1, which can be associated with the proteasome through Rad23, removes the *N*-glycans of glycoproteins before proteasomal degradation (adapted from ref. 187).

targeting. Subsequent degradation of ERAD substrates by the proteasome implies their transport from the ER to the cytosol, a phenomenon named retrotranslocation (**Figure 13**).

ERAD-L and ERAD-M substrates are first recognized by specific ER chaperones, such as BiP (the mammalian homologue of yeast Kar2) or protein disulfide isomerase (PDI), which were shown to prevent soluble substrate aggregation to ensure efficient retrotranslocation (184). Misfolded ER proteins that contain *N*-glycosylations are also recognized and targeted for ERAD by chaperone-like lectins, such as ER degradation enhancing α -mannosidase-like (EDEM) proteins or OS-9 (the mammalian homologue of yeast Yos9) (185). These ER lectins are thought to link ERAD substrates to the membrane-associated ubiquitylation machinery. Indeed, Yos9 was found to be associated with Hrd3, a partner of the Hrd1 E3 ubiquitin ligase, which is responsible for the ubiquitylation of ERAD-M and ERAD-L substrates prior to their degradation (183, 186). Differently, degradation of ERAD-C substrates, which expose misfolded domains to the cytosol, relies on the intervention of cytosolic chaperones of the Hsp70/Hsp40 system and requires the E3 ubiquitin ligase Doa10 for polyubiquitylation (185). In some instances, however, Hrd1 and Doa10 can have overlapping specificities. Specific E2 Ub-conjugating enzymes, such as Ubc6 and Ubc7, are found to interact with these E3 ligases on the cytosolic side of the ER membrane (187). The catalytic sites of the membrane-associated E3 ligases implicated in ERAD pathway are also located in the cytoplasm. This implies that ER luminal substrates gain access to the cytoplasm to be polyubiquitylated. Indeed, a critical, minimal length of the polyubiquitin chain seems to be imperative for retrotranslocation (188). Derlin-1, the mammalian homologue of the yeast Der1, was also found to be required for dislocation of misfolded proteins from the ER and is believed to be part of the retrotranslocation complex (189). Sec61, which is a component of the translocation channel for newly synthesized proteins in the ER, is also believed to be a component of the retrotranslocation channel for antigenic peptides that fail to bind to MHC molecules (190) and for ERAD-L substrates (191, 192). However, two recent in vitro studies using yeast components argued against a role of Sec61 in this process. Using in vitro fluorescence and photo-crosslinking approaches, the requirement of Der1, rather than Sec61, was demonstrated for the retrotranslocation of two different ERAD-L substrates (193). A central role of the E3 ligase Hrd1 in the retrotranslocation complex was proposed by showing that Hrd3, Der1 and Yos9 are involved in substrate delivery to Hrd1, whereas Usa1 (see figure 13) serves both to recruit Der1 to Hrd1 and to induce Hrd1 oligomerization (186). The exact composition of the retrotranslocation complex in mammalian cells remains now to be elucidated.

The extraction of ubiquitylated substrates from the ER prior to their degradation is driven by the yeast Cdc48 complex (p97 in mammals), which is a hexameric AAA ATPase that is thought to

couple ATP hydrolysis with polypeptide retrotranslocation (188, 194, 195). The Cdc48 complex might be recruited to the ER membrane in part through its interaction with the Ub-regulatory X (UBX) domain of Ubx2 in yeast, or with valosin-containing protein-interacting membrane protein (VIMP) in mammals (196, 197). Biochemical studies have shown that Cdc48/p97 extracts ubiquitylated substrates either by actively pulling a polypeptide through the retrotranslocation channel and/or lipid membrane, or by segregating a polypeptide that has already been liberated from the ER membrane (188, 194, 195). Nevertheless, it is not completely clear whether the intervention of Cdc48/p97 complex is essential for the extraction all ERAD substrates from the ER. In fact, a recent study has suggested that the extent to which a membrane protein is embedded in the lipid bilayer might dictate the degree to which Cdc48/p97 function is needed (198).

Cdc48/p97 then delivers substrates to the proteasome via Ub-associated (UBA) and UBX domain-containing molecules, such as Dsk2 and Rad23 (199, 200). Rad23 was found to interact both with the proteasome and with ubiquitylated substrates, but also with the Cdc48/p97 complex, thus indirectly linking proteasomes to Cdc48/p97 (201). The requirement of Cdc48/p97 for the delivery of all ERAD substrates to the proteasome is also discussed. First, it was shown that a substantial percentage of proteasomes reside at the surface of the ER membrane, thus ideally positioned to receive and degrade ERAD substrates (202). Proteasomes were later shown to be sufficient to retrotranslocate and degrade an ERAD substrate in vitro, these two activities being able to be uncoupled: the 19S RP alone drives ATP-dependent retrotranslocation of the substrate, and addition of the 20S CP catalyses its subsequent degradation (203). In addition, 26S proteasomes were found to directly bind to Sec61 in the ER membrane (204). This interaction seemed to be mediated by the base of the 19S RP, suggesting that the Rpt subunits, which are responsible for substrate translocation into the proteolytic core of the proteasome, may also be able to extract misfolded proteins from the ER through the retrotranslocation channel. This hypothesis is supported by the structural and functional analogies shared by Cdc48/p97 and proteasomal Rpt subunits, which both form a hexameric ring of AAA ATPases. A recent study strengthens this hypothesis by defining which Rpt subunits are directly involved in retrotranslocation (205). Rpt4 was shown to be necessary for the dislocation of an ERAD substrate and appeared therefore to have overlapping functions with Cdc48/p97.

The deglycosylating enzyme **peptide N-glycanase** (PNGase, or Png1 in yeast), which is present in the cytosol, is implicated in the degradation of misfolded glycosylated ERAD substrates (206, 207). PNGase removes N-linked glycans by hydrolysing the β -glycoamide bond between an Asn

residue and the first *N*-acetylglucosamine of the oligosaccharide, resulting in the release of a free glycan and the concomitant conversion of the Asn into Asp (208). Consistent with its implication in ERAD and in proteasomal degradation, PNGase was found to interact with Derlin-1, Cdc48/p97, components of the ubiquitylation machinery and with the 19S RP subunit Rpt1 through Rad23 (209-214). One model based on these studies suggested that PNGase is recruited to the 26S proteasome and removes the *N*-linked glycans from the substrate prior to its proteasomal degradation. The observation that the *N*-linked glycans can act as a recognition signal for an E3 Ub ligase complex placed PNGase downstream of ubiquitylation and upstream of proteasomal degradation (215). However, studies using yeast deleted for *Pngl* gene or mammalian cells in which PNGase expression was silenced by small interfering RNA (siRNA), showed only a minor delay in the degradation of glycosylated proteins (207, 216). Since both yeast and mammalian cells have a single gene coding for cytoplasmic *N*-glycanase activity, an alternative model in which PNGase activity is not a prerequisite for proteasomal degradation was raised. In agreement, it was observed that inhibition of PNGase deglycosylation by the peptide inhibitor z-VAD-fmk did not impede proteasomal degradation of glycosylated substrates in living cells (217). The degradation of *N*-linked ribonuclease B (RNaseB) and its deglycosylated form were assessed in vitro with purified 26S proteasomes (218). This study showed that although the oligosaccharide moiety did not impede the degradation of RNaseB, it contributed to slow the degradation rate and altered the repertoire of the digestion products containing the glycosylation site. Interestingly, the authors of this study also observed that the presence of a *N*-linked glycan near a MHC class I epitope affects its presentation, which is diminished when PNGase is inhibited. Together, these results demonstrated that glycosylation alters the pattern of proteasomal cleavage, at least in vitro. In line with this study, another demonstrated that tyrosinase molecules can be degraded by the proteasome while they still bear *N*-glycans (219). However, the authors observed that the degradation of glycosylated tyrosinase molecules gave rise to a set of discrete intermediate products that were not detected when unglycosylated molecules were degraded. They showed that these discrete intermediate products were glycosylated and were detected only when proteasomal activity was compromised. Consistent with these observations, they proposed a model in which glycosylated tyrosinase molecules enter the catalytic chamber in a hairpin conformation at some distance from any glycosylation site. The proteasome would degrade these molecules until it encounters a *N*-glycan chain and would then release the remainder of the molecule. This remainder could be subsequently degraded by the same or other proteasomes in the same manner, giving rise to a set of discrete intermediates

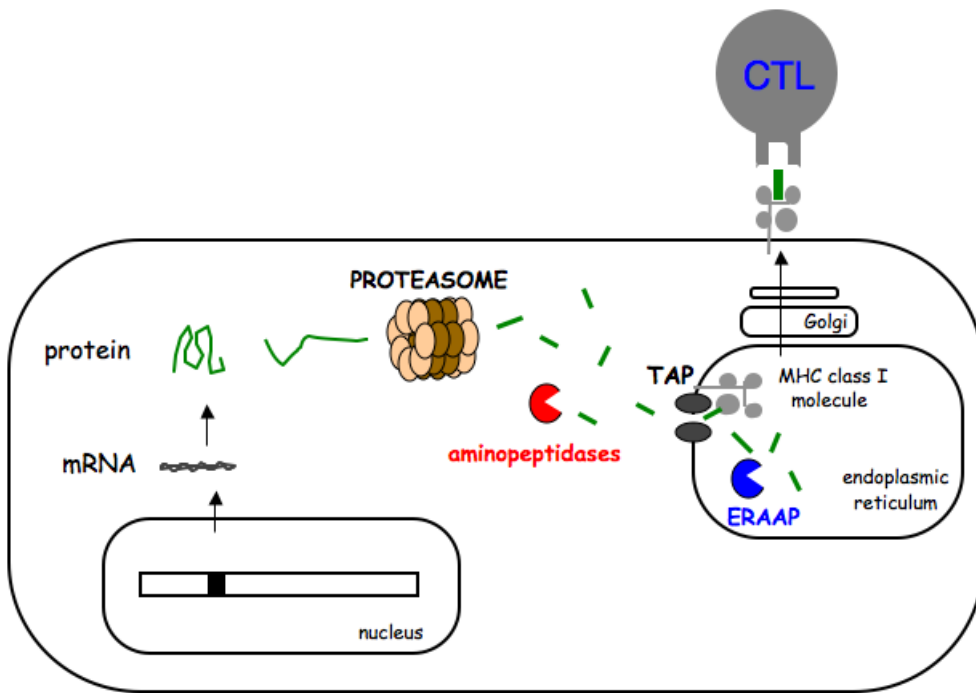


Figure 14. Processing pathway of MHC class I antigenic peptides.

Intracellular proteins are degraded by the proteasome into peptides of various lengths that can be N-terminally trimmed by cytosolic aminopeptidases. These peptides are further transported into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). ER-associated aminopeptidase (ERAAP) can also trim N-extended peptides to adjust their size for efficient binding to MHC class I molecules. Peptide-loaded MHC molecules are then translocated, through the Golgi apparatus, to the cell surface. They are recognized by specific cytotoxic T lymphocytes (CTL) whose activation can eventually lead to the destruction of the target cell.

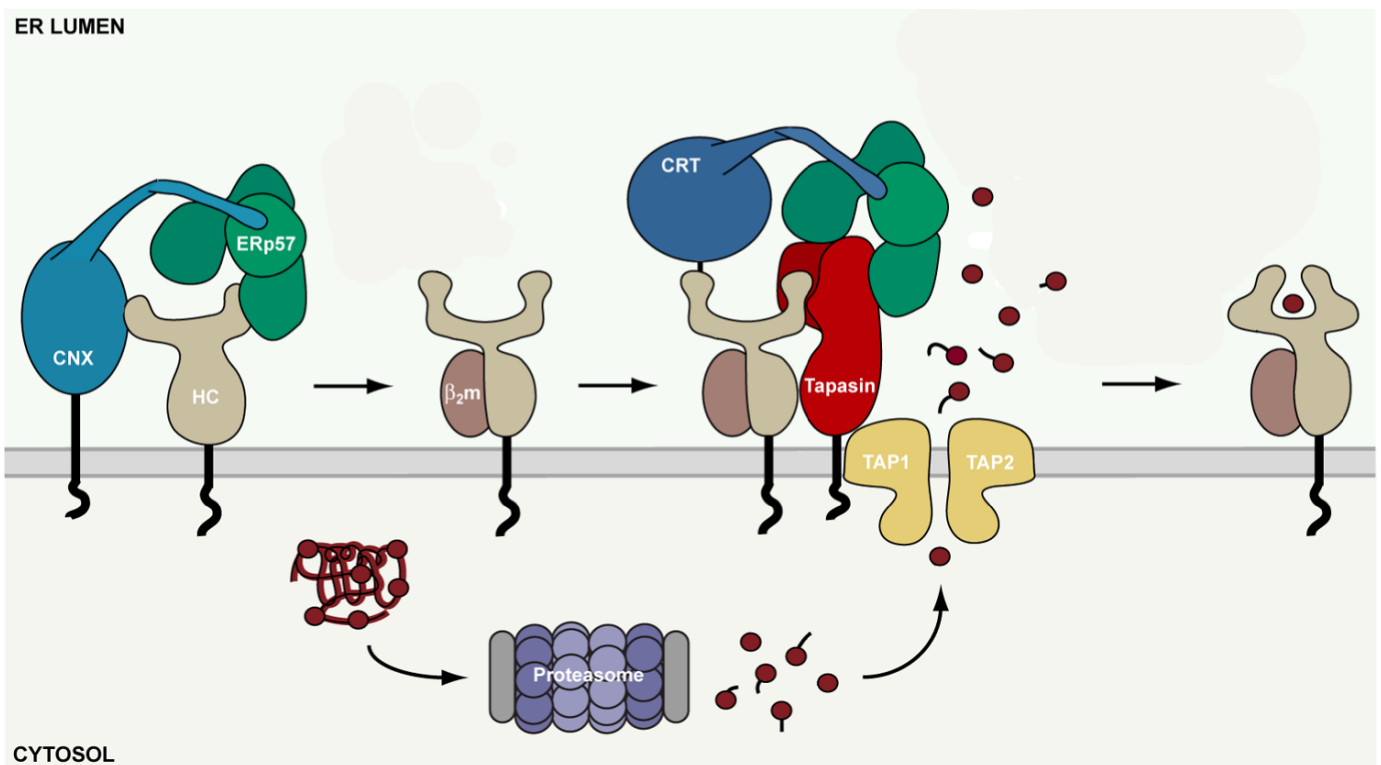
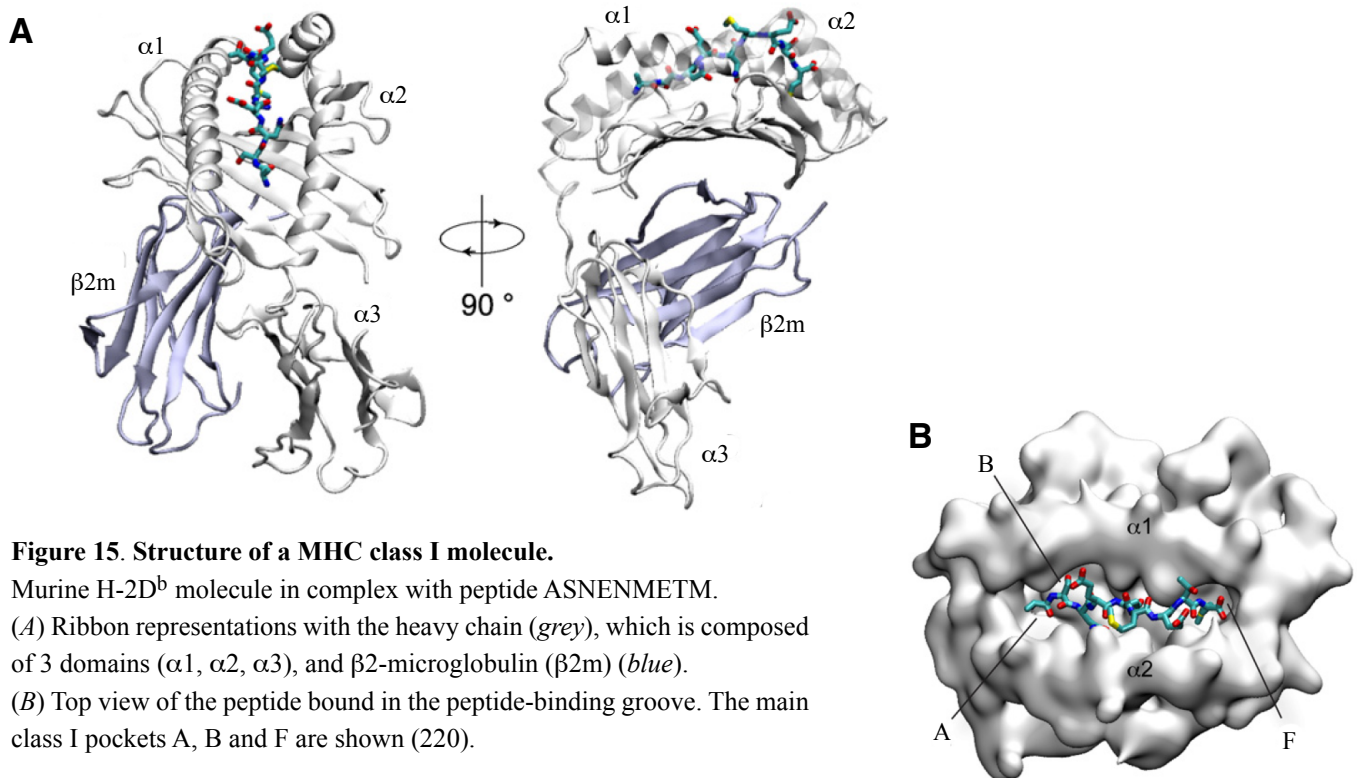
with *N*-glycosylated residues in proximity to one terminus or the other. Further studies will be needed to confirm, or not, this mechanism.

II) Processing of MHC class I antigenic peptides

The immune system utilizes the two principal proteolytic systems involved in protein turnover to produce antigenic peptides: the lysosomal/endosomal apparatus is used for the production of MHC class II-presented peptides from extracellular proteins and the ubiquitin-proteasome system is responsible for the production of MHC class I peptides from intracellular proteins (**Figure 14**). MHC class I molecules present antigenic peptides at the cell surface, so that they can be recognized by specific T cell receptors (TCR) present on CD8⁺ cytotoxic T lymphocytes (CTL). By doing this, these T cells can monitor the organism for evidence of danger, caused by pathogen intrusion or by malignant transformation. MHC class I ligands are usually peptides of 8-11 amino acids in length. The generation of these peptides therefore requires the degradation of proteins into fragments of precise size. Although the proteasome is the major proteolytic system producing these antigenic peptides, other proteases significantly contribute to the production of the peptide pool, essentially by catalyzing the N-terminal trimming of proteasomal products to the correct size. Proteasomal products are usually found in the cytosol, whereas MHC class I molecules are synthesized in the ER. Thus, peptides have to gain access to the ER to be loaded onto MHC molecules. This function is mediated by the TAP transporter, which is a large protein complex present in the ER membrane and belongs to the ATP-binding cassette transporter family. In the ER, peptides can be further trimmed by the ER-associated aminopeptidase (ERAAP), which helps adjusting the size of peptides for binding to MHC class I molecules.

1. MHC class I molecules and peptide-loading complex

MHC class I molecules are cell surface glycoproteins presenting antigenic peptides to CD8⁺ T lymphocytes. They are expressed on all nucleated cells. With three major loci encoding different MHC class I molecules in both mouse and human, each heterozygous individual can express up to six different class I molecules. These loci are highly polymorphic so that various alleles exist



at different frequencies. In human, the three types of MHC class I molecules are HLA-A, HLA-B and HLA-C, and in mouse H-2K, H-2D and H-2L molecules.

MHC class I molecules consist of two non-covalently bound subunits: a polymorphic 45-kDa transmembrane heavy chain (HC) and a soluble 12-kDa light chain termed β 2-microglobulin (β 2m) (**Figure 15A**) (220). MHC class I molecules are translated and assembled in the ER (**Figure 16**) (221). The newly synthesized HC rapidly binds to the transmembrane lectin-chaperone calnexin and associated ERp57, which is a thiol oxidoreductase responsible for disulfide bond formation (222). Upon association of β 2m with HC, calnexin is replaced by its homologue calreticulin. The newly assembled HC- β 2m heterodimers are unstable and required entry into the peptide-loading complex (PLC) for stability. The PLC consists of a HC- β 2m heterodimer, calreticulin, ERp57, tapasin and TAP transporter. It serves to transport peptides across the ER membrane and facilitates the loading of appropriate peptides onto class I molecules. Tapasin has a central role in the PLC by interacting with TAP and the HC, but also with ERp57 and is thought to optimize the binding of peptides to MHC molecules.

Peptides are closely associated with the MHC molecules by specific non-covalent interactions in the peptide-binding groove, which forms a cleft located between α -helices of the HC (**Figure 15**). The amino acid sequence corresponding to this HC region varies from allele to allele, thus changing the specificity of the peptide-binding groove. The cleft is closed at both ends, limiting the size of suitable peptides to 8-10 amino acids (223). Peptides are held by hydrogen bonds at the N and C termini, and by binding to the specificity pockets which anchor the peptide in the groove (224). One anchor residue is always located at the C terminus of the peptide and fits into the F pocket. The other one or two anchor residues are usually located near the N terminus and fit into A and B pockets. To allow peptide binding, the anchor residue positions must be occupied by specific residues defined by the MHC class I haplotype. After optimal peptide binding, MHC molecules are released from the PLC, exit the ER and are transported in vesicles that fuse with the ER-Golgi intermediate compartment and traverse the Golgi apparatus to the cell surface.

2. Sources of class I antigenic peptides

Production of class I antigenic peptides can be considered as a by-product of general protein turnover in the cytosol, which is usually mediated by the proteasome. Each cell therefore presents a complex mixture of peptides, estimated in the hundreds to thousands, from proteins synthesized within that cell. However, the question of how the proteasome can gain sufficient access for antigen processing of low-abundance, metabolically stable or compartmentalized proteins is not fully resolved. An intriguing observation is that cells could sample most proteins after their synthesis rather than after their turnover (225, 226). This timing allows presentation of viral gene products early after infection and presents the advantage of capturing proteins before they transit to various subcellular compartments or cell surface, away from the antigen processing machinery. A theory was therefore proposed that a rapidly degraded subset of proteins that do not accumulate intracellularly was the major source of MHC class I peptides (225). These defective ribosomal products (DRiPs) are proteins targeted for degradation as a result of premature termination or misfolding and could account for the production of substantial quantities of peptide-MHC complexes in the absence of detectable accumulation of protein antigen, enabling rapid immunosurveillance of viral and cellular translation products. Although it was clearly demonstrated that 30% of newly synthesized proteins were immediately degraded (227), a high error rate of protein production is not crucial to validate the DRiP model in antigen processing, which still remains controversial (228). The DRiP model was further extended by proposing that cells possess a specialized machinery, possibly in the form of "immunoribosomes", to couple protein synthesis to antigen presentation (229). Specialized ribosomes with direct access to presumed TAP-interacting proteasomes would translate a subset of mRNAs dedicated to antigenic peptide production. The immunoribosome hypothesis would explain the close linkage between translation and generation of class I peptide ligands described in many systems. It predicts that peptides generated from DRiPs are protected from competition for class I presentation by cytosolic peptides generated from other processes, a prediction recently supported by another study (230). Antigen processing appears to be compartmentalized in a manner that prevents abundant cytosolic peptides from blocking presentation of peptides derived from DRiPs. Thus, it would be divided in at least two functional compartments, one for peptides derived from DRiPs and the other for cytosolic peptides generated from ubiquitylated proteins. This compartmentalization of antigen processing would also explain the highly efficient generation of MHC class I-peptide complexes from very small amounts of exogenous antigens delivered to the cytosol (231).

In addition to conventional translation products that can nevertheless end up as DRiPs, cells can also generate class I peptides from cryptic translational products, which are polypeptides that are synthesized in cells by unconventional translational mechanisms. These include peptides encoded by introns, intron/exon junctions, 5' and 3' untranslated regions, and alternate translational reading frames (232). One antigenic peptide was also found to result from the reverse strand transcription of a gene (233).

Most peptides for MHC class I presentation are thought to be derived from proteasomal breakdown of cytosolic proteins, including DRiPs, cryptic translational products but also ERAD substrates (§ I-6). However, other sources of class I antigenic peptides were mentioned. Dendritic cells (DC), for example, deliver peptides derived from endocytosed material to the MHC class I pathway. This phenomenon is termed “cross-presentation” and will be presented later (§ II-6).

3. The ubiquitin-proteasome system in MHC class I antigen processing

3.1. Evidence for the implication of the UPS

Initial evidence that the UPS is the principal machinery providing peptides presented on MHC class I molecules came through studies showing that ubiquitylation is necessary for the presentation of some class I antigenic peptides (234-236). However, ubiquitylation is not absolutely required for the generation of all class I antigenic peptides, since many substrates can be degraded by the proteasome without prior ubiquitylation (§ I-3).

The role of the proteasome in the production of antigenic peptides was extensively substantiated. A first hint came from the discovery that both proteasomal catalytic subunits $\beta 1i$ and $\beta 5i$ were encoded in the MHC locus, and that the incorporation of these subunits into proteasomes enhanced the presentation of some antigens (§ I-4.1 and II-3.3). In addition, about 30% of the peptide fragments produced by the proteasome are 8-9 residues or longer, and might therefore correspond to antigenic peptides or precursors of antigenic peptides (90). However, the strongest evidence for the role of the proteasome in antigen presentation was the demonstration that proteasome inhibitors selectively blocked the production of the majority of class I antigenic peptides in cells. Indeed, peptide aldehydes and lactacystin completely blocked the presentation of peptides derived from a wide variety of cellular or viral proteins (16, 172, 237). In contrast,

blocking proteasome function did not impair the presentation of antigenic peptides when these were expressed from a minigene or introduced inside the antigen-presenting cell by electroporation, demonstrating that proteasome inhibitors block antigen presentation by interfering with the proteolytic generation of the presented peptides. Later, the use of proteasome inhibitors displaying higher specificity, such as epoxomicin, further confirmed the implication of the proteasome in antigen presentation (238).

3.2. Role of the proteasome in the production of antigenic peptides

If studies using proteasome inhibitors have demonstrated the implication of proteasomes in the production of antigenic peptides, the nature of the enzymes involved in the generation of the correct N- or C-terminal end of the peptide still needed to be investigated. To do so, proteasome inhibitors were used to study the production of peptides from C-terminally extended precursors (239-241). Proteasome inhibitors were able to block presentation of antigenic peptides from precursors containing a C-terminal extension of only a single residue. It was therefore generally admitted that the proteasome was responsible for the production of the C terminus of class I antigenic peptides. On the other hand, proteasome inhibitors did not always block the presentation of peptides derived from N-terminally extended precursors, even when extensions were as long as 25 residues, suggesting that the proteasome is not responsible for the generation of the correct N terminus of all antigenic peptides. Because acetylation almost completely blocked the N-terminal trimming of some precursor peptides in cellular extracts but not by purified proteasomes, it was suggested that in some cases, N-terminal trimming involves a non-proteasomal aminopeptidase activity (240). The role of non-proteasomal peptidases will be detailed in a following paragraph (§ II-4).

Several in vitro studies using purified 20S proteasomes and long synthetic precursor peptides tempted to clarify more precisely the contribution of the proteasome to epitope generation. In such studies, many cleavage products were detected, some producing the antigenic peptide (92, 242, 243), while others occur within the sequence of the epitope, destroying the antigenic peptide (244-246). Cleavages occurring outside the epitope sometimes produced peptides that may serve in vivo as precursors for presentation. However, these in vitro studies might not exactly represent what happens in living cells, particularly because they used polypeptides as substrates and not full-length proteins, but also long non-linear incubations in which the initial proteasome products can re-enter the particles and undergo additional rounds of hydrolysis, a process which is unlikely to occur in vivo (§ I-3). These facts were taken into account in a detailed study in which

denatured ovalbumin molecules were digested with purified 20S and 26S proteasomes at linear rates, producing a set of different peptides also at linear rates (123). Degradation of ovalbumin led to the production of the immunodominant peptide SIINFEKL, but also produced N-extended precursors of this peptide that might be further trimmed in vivo to the mature antigenic peptide. Some C-terminally-extended precursors were also detected in the digestion mixture, however they were found to be produced at much higher rates by 20S proteasomes than by 26S proteasomes. This is consistent with previous studies showing that 20S and 26S proteasomes, despite their identical composition in proteolytic active sites, could cleave proteins at different sites (90, 95).

Although proteasomes are required for antigen presentation, they destroy many more peptides than they generate. The analysis of size distribution of proteasomal products revealed that only a minor fraction of peptides (~15%) presents the correct size for binding to MHC class I molecules, and ~70% are too short (90, 122). Furthermore, degradation of ovalbumin by purified proteasomes indicated that the immunodominant peptide SIINFEKL is degraded more than 90% of the time (123). The production of antigenic peptides by proteasomes therefore seems to be a stochastic and inefficient process.

3.3. Role of the immunoproteasome in antigen processing

In the presence of IFN γ , three additional proteasome catalytic subunits (β 1i, β 2i and β 5i) are induced (§ I-4.1). Two of these three subunits are encoded in the MHC locus, together with other genes related to the immune response. The incorporation of the immunosubunits into the proteasome alters peptidase activities by stimulating cleavages after hydrophobic and basic residues, and by decreasing cleavages after acidic residues. Because the TAP transporter and MHC molecules prefer peptides bearing hydrophobic or basic residues at their C terminus, the immunoproteasome was thought to increase the production of antigenic peptides. Consistent with these assumptions, transfection of immunosubunits enhanced the presentation of several antigenic peptides (247-249), while the deletion of their genes in mice suppressed the presentation of some epitopes (248, 250). The first direct evidence that the immunoproteasome can enhance the presentation of antigenic peptides was provided by the in vitro study of the degradation of ovalbumin by purified proteasomes (123). The authors found that the total amount of SIINFEKL-containing peptides produced per ovalbumin molecule degraded was significantly higher with immunoproteasomes than with standard proteasomes. Surprisingly, this

increase was not due to a greater production of antigenic peptide SIINFEKL, but was due to a greater production of N-extended precursors. Recent work using mass spectrometric analyses of primary DCs expressing or not immunosubunits contributed to provide a more general view by showing that immunoproteasomes substantially increase the abundance and diversity of MHC class I-associated antigenic peptides (251).

The formation of immunoproteasomes is induced by IFN γ early after viral infection (252). The influence of immunoproteasomes on antigen presentation was therefore largely studied in the context of viral infection. The generation of a large number of viral epitopes of various origins was analysed in different in vitro studies and, in the majority of cases, the presence of immunoproteasomes has a positive effect on MHC class I presentation (238, 248, 250, 253). These results were supported by studies in which the production of immunodominant viral peptides was favored in infected cells expressing the immunosubunits (254, 255), and by an in vivo study in which β 2i-deficient mice displayed an altered T cell repertoire against lymphocytic choriomeningitis virus (LCMV) epitopes (256). Immunoproteasomes were also shown to influence immunodominance hierarchies of antiviral CD8⁺ T cells by producing different amounts of given epitopes, compared to standard proteasomes (257). Thus, it was expected that T cells directed against antigenic peptides produced by the immunoproteasome would dominate immune responses. However, this notion was challenged by another study which demonstrated that mice lacking β 1i and β 5i subunits were still able to induce efficient CTL responses against LCMV infection (258).

Although viral epitopes appeared to be processed more efficiently by immunoproteasomes than by standard proteasomes, this was not the case for tumor antigenic peptides derived from self-proteins. Indeed, several human antigenic peptides expressed on tumor cells were found to be more efficiently, and sometimes exclusively, produced by the standard proteasome (244, 259, 260). The processing of antigenic peptides by standard proteasomes and immunoproteasomes was studied in detail for three examples of tumor peptides and revealed that the differences observed mainly resulted from differences in destructive cleavages within the sequence of the epitope (244).

Altogether, these data draw a picture in which self and differentiation epitopes are poorly processed by immunoproteasomes, whereas viral epitopes are more efficiently processed by these IFN γ -inducible proteasomes than by constitutive proteasomes. An extensive study of antigen processing that would include the recently characterized intermediate forms of the proteasome, which are found in many tissues (§ I-4.1), may abrogate this dichotomy by

revealing additional specificities (131). Importantly, two tumor antigens were already shown to be processed exclusively either by the intermediate $\beta 5i$ -proteasome or by the $\beta 1i$ - $\beta 5i$ -proteasome. Beyond their contribution to antigen processing, immunoproteasomes were recently found to have a crucial role in antiviral humoral and innate immune responses, at least in mice (261).

3.4 Role of PA28 in antigen processing

Concomitant with the synthesis of immunosubunits and other components of the MHC class I presentation pathway, IFN γ stimulates the expression of both PA28 α and β subunits (§ I-4.2). In contrast to the immunosubunits, PA28 is expressed at a basal level in all cell types of higher eukaryotes and increased constitutive levels are observed upon DC maturation (262, 263). These observations led to the assumption that PA28 might have a function in antigen processing. Cell systems that express both PA28 subunits independently of IFN γ were used to study the role of PA28 in antigen processing. In this context, it was shown that PA28 enhances the presentation of several viral epitopes without increasing the overall protein turnover, whereas the presentation of other virus-derived epitopes was not affected (248, 264, 265). Enhanced peptide presentation was independent of the presence of the immunosubunits in proteasome particles. Furthermore, the absence of PA28 in vivo reduced epitope processing and CTL recognition of some antigenic peptides, as shown in PA28-deficient mice (266). One epitope, derived from melanoma differentiation antigen TRP2 (tyrosinase-related protein 2), was found to be dependent on the expression of PA28 (267). The lack of presentation of this epitope by a melanoma cell line correlated with a strongly impaired expression of PA28 subunits in these cells. Presentation of this epitope could be rescued by transient transfection of cDNAs coding for PA28 subunits. In vitro experiments with purified 20S proteasomes, PA28 and a synthetic precursor peptide confirmed the results obtained with melanoma cells, and revealed that the presence of PA28 was indeed required to produce the TRP2 epitope and an N-terminally extended precursor peptide. Interestingly, the PA28-dependence of TRP2 epitope was further shown to rely on its flanking sequences (268). Indeed, its proximal N-terminal flanking residues are sufficient to permit PA28-induced coordinated dual cleavages, which were previously observed in other studies (138, 140). These results therefore support the notion that binding of PA28 to the proteasome not only opens the gate but also induces structural changes into the 20S CP (§ I-4.2). It should be noted that this example of PA28-dependent epitope is unique and does not allow one to conclude that PA28 plays a primordial role in MHC class I antigen presentation. However, a recent study

showed that the effects of PA28 on antigen presentation were different depending on the allelic polymorphism of murine MHC class I molecules (269). Indeed, whereas the production of many K^d ligands was impaired in cells expressing PA28, the production of most K^b and L^d epitopes was augmented. Consistent with these results, the cell surface expression of K^d molecules was decreased, while K^b and L^d molecules were up-regulated in the presence of PA28. These results were supported by in vitro experiments demonstrating that purified PA28-proteasome complexes were unable to produce some K^d epitopes from longer precursors, whereas K^b ligands were efficiently produced by the same proteasome preparations. However, the origin of the discrepancy between these MHC class I alleles could not be identified in this study. Whether these findings also apply to the processing of human antigens still needs to be addressed.

4. Cytosolic proteases involved in MHC class I antigen processing

Because proteasome inhibitors sometimes failed to block the processing of some antigenic peptides, the role played by additional proteolytic enzymes in this process has been investigated. As stated previously, N-terminal trimming seems to be mediated by aminopeptidases since this trimming is inhibited by acetylating the N-terminal amino group of the N-extended precursors (§ II-3.2). Furthermore, *o*-phenanthroline, which blocks all metalloproteases, and bestatin, an inhibitor of many metallo-aminopeptidases, were also shown to markedly inhibit this trimming process (270). Cells contain many cytosolic peptidases whose principal function is to rapidly hydrolyse the proteasomal products in order to maintain the cellular pool of amino acids necessary for protein synthesis. However, a large number of findings have implicated some of these cytosolic peptidases in the processing of class I antigenic peptides (271-275).

One peptidase that has been extensively studied in antigen processing is **tripeptidyl peptidase II (TPPII)**. This cytosolic serine protease can trim peptides longer than 15 amino acids, whereas most other peptidases only cleave shorter peptides. This is a very large (~6 MDa), self-compartmentalized peptidase composed of identical 130-kDa subunits assembled in a multimeric spindle structure (276). TPPII functions as an exopeptidase, removing three N-terminal residues, but also exhibits a weaker endoproteolytic activity with trypsin-like specificity (175). At first, TPPII was found to be essential for the production of a human immunodeficiency virus epitope (277). The presentation of this epitope was strongly impaired in cells after inhibition or silencing of TPPII, whereas no effect was observed after proteasomal inhibition. In this study, although TPPII was able to produce the correct N and C termini of the epitope in vitro, the degradation of

the parental protein inside cells was not addressed. Since proteasome inhibitors do not entirely block all proteasomal activities, it remained unknown whether TPPII produced this epitope in combination with or independently of the proteasome. Although initial reports suggested that TPPII was required for the generation of many antigenic peptides (278, 279), subsequent studies using chemical inhibitors, RNA interference, and TPPII-deficient mice found no major defect in antigen presentation (280-284). Therefore, although TPPII seems to be required for the N-terminal trimming of several epitopes, its activity does not appear essential for the processing of the majority of class I antigenic peptides.

The other cytosolic peptidases that were shown to be involved in the N-terminal trimming of antigenic peptides are **leucine aminopeptidase** (LAP), **bleomycin hydrolase** (BH) and **puromycin-sensitive aminopeptidase** (PSA). LAP is a metallo-aminopeptidase with broad substrate specificity and wide tissue distribution. Interestingly, LAP is inducible by IFN γ and its overexpression in cells was associated with a more rapid trimming of precursor peptides to the mature epitope (270). However, a more recent report using LAP-deficient mice demonstrated that this peptidase is not essential for trimming peptides or generating MHC class I epitopes (285).

Similarly, PSA and BH were initially shown to be required for the trimming of certain N-extended precursors of epitopes in cytosolic extracts (286, 287), but further studies with mice deficient for these two aminopeptidases showed no defect in producing and presenting mature epitopes from N-extended precursors, or in antigen presentation in general (288, 289). These results may indicate a substantial redundancy among these different cytosolic enzymes, so that trimming is not reduced by the loss of only one or two of these peptidases. This would be consistent with their broad substrate specificity.

Thimet oligopeptidase (TOP) is a metallo-endopeptidase that was found to be associated with antigenic peptide degradation in the cytosol. Indeed, TOP inhibition protected antigenic peptides from degradation in cytosolic extracts (290, 291). Furthermore, TOP overexpression reduced the presentation of three model epitopes and the cell surface expression of MHC class I molecules (292). Conversely, downregulation of TOP enhanced presentation of a model epitope and increased total class I molecule expression. These observations were recently confirmed in a study analysing the role of TOP and **nardilysin**, which is also a cytosolic metallo-endopeptidase, in antigen processing (161). The authors showed that overexpression of TOP resulted in 20% lower expression of MHC class I molecules, whereas overexpression of nardilysin led to

modestly attenuated expression of these molecules. However, by studying the processing of several epitopes, they demonstrated that TOP could function as C-terminal peptidase, and nardilysin could contribute to both the C-terminal and N-terminal trimmings of epitope precursors in vitro. The use of siRNAs targeting these two peptidases confirmed their implication in cells expressing these epitopes. Because of their substrate length constraints of about 30 amino acids and 17 amino acids, respectively, nardilysin and TOP probably act only after protein hydrolysis by the proteasome.

The aminopeptidase **DPP9** (for dipeptidyl peptidase-9), which is rate-limiting for the degradation of proline-containing peptides by cleaving X-Pro dipeptides, was shown to decrease the presentation of an antigenic peptide with a proline residue at position 2 (293). Hence, the authors of this study proposed that DPP9 could have a negative role in the production of antigenic peptides containing a X-Pro motif at their N terminus, and, in contrast, a positive role for other peptides if a X-Pro dipeptide flanks the N terminus of the mature epitope.

Notwithstanding the crucial role of the proteasome in protein degradation and antigen processing, an elegant study recently reported the production of a tumor antigenic peptide by the metallo-endopeptidase **insulin degrading enzyme** (IDE) (294). Interestingly, degradation of MAGE-A3 protein, from which this epitope is derived, was significantly slowed down in cells treated either with a proteasome inhibitor or a metalloprotease inhibitor, and was completely inhibited after treatment with both inhibitors. This observation, together with the existence of distinct MAGE-A3 epitopes, which are produced by the proteasome, suggested that two parallel degradative pathways involving proteasomes or IDE, could lead to the production of different sets of antigenic peptides. Another recent study, albeit less documented, described a contributing role of caspases to class I antigen presentation in virus-infected cells (295).

5. ER-associated aminopeptidase (ERAAP)

A small fraction of the peptides produced by proteasomes escape destruction in the cytosol and are transported by TAP into the ER. TAP transports peptides whose size ranges from 8 to 20 amino acids and can therefore import into the ER both mature epitopes and longer precursors (296). In some cases, N-extended cytosolic precursors have a higher affinity for the TAP transporter than mature epitopes so that they are more rapidly transported into the ER and sometimes constitute the major pool of peptides at the base of antigen presentation (297). These

observations strongly suggested that these precursors could be trimmed in the ER. Experimental evidence of an ER aminopeptidase activity came from a study in which N-extended peptides were directly expressed in the ER of TAP-deficient cells using a signal sequence. Their extra N-terminal residues were removed and the peptides were presented on MHC class I molecules (298). This peptidase activity was later identified and attributed to two IFN γ -inducible metallo-aminopeptidases in humans and one in mice (299-301). They were given several different names, but they are now named ERAP1 and ERAP2 in humans and ERAAP in mice. ERAP1 and ERAP2 are highly homologous and present 50% of sequence identity. They can form heterodimeric complexes and may act in a concerted fashion to remove longer and heterogeneous extensions from precursor peptides (301).

Because ERAP1 rapidly trims peptides to the size of 8-9 amino acids, it was considered as a specialized peptidase involved in the generation of peptides with adequate size for presentation on MHC class I molecules (299). However, by trimming ~50% of 9-mer peptides to 8-mers, ERAP1 does not always generate mature epitopes, but can also destroy some antigenic peptides and limit their presentation. Nevertheless, it has been suggested that ERAP1 might trim the protruding end of long peptides when they are bound to MHC class I molecules (302), an hypothesis that was refuted by showing that an N-extended 15-mer precursor peptide was no longer trimmed by ERAAP when associated with the MHC class I-presenting molecule (303).

ERAP1 and ERAP2 present different cleavage specificities according to the use of fluorogenic peptide substrates: ERAP1 preferentially removes hydrophobic residues such as leucine, while ERAP2 has a strong preference for the basic residues arginine et lysine (301). When antigenic peptide precursors were used as substrates, these enzymes were less selective and could cleave after most amino acids, except after or before proline residues (300, 304). An extensive study on the specificity of ERAP1 in different cell lines recently confirmed these findings, and failed to identify a simple chemical feature that would determine whether a particular amino acid renders a peptide a good substrate or not (305). Trimming by ERAP1 may also be affected by the internal peptide sequence, and it was even suggested that the C-terminal residue may have an impact on this trimming (306).

Several studies reported an important role of ERAP1 in MHC class I antigen presentation. ERAP1/2-deficient cells were unable to cleave N-extended peptides in the ER, strongly suggesting that these enzymes represent the only trimming activity in this compartment (300). Furthermore, ERAP1-deficient cells have reduced surface levels of MHC class I molecules and the peptide-MHC complexes produced are less stable than on wild-type cells (307, 308).

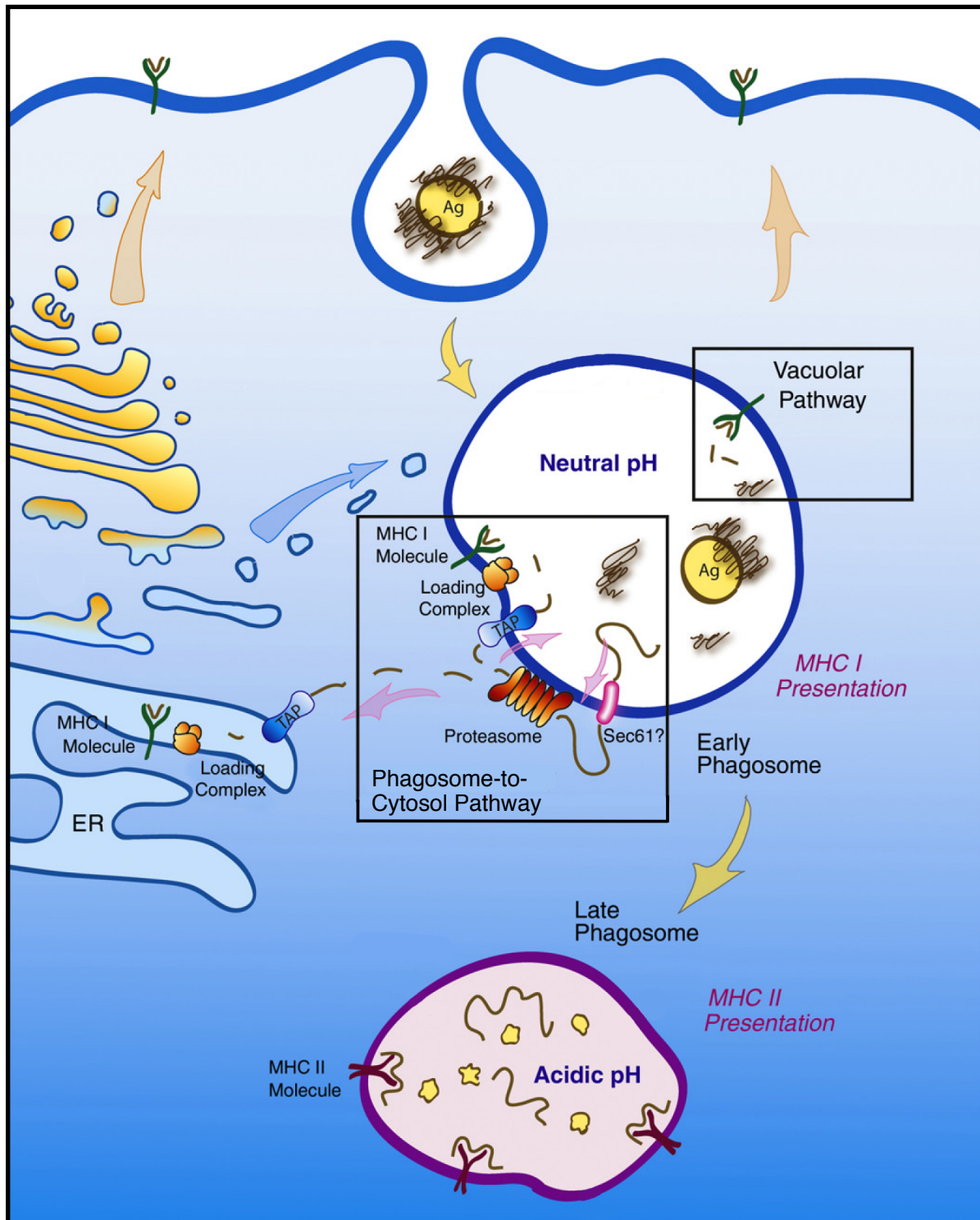


Figure 17. Major pathways for cross-presentation.

Early endocytic compartments are enriched in certain ER compounds and display neutral pH and low degradation environment (as compared to later compartments). These are most probably the compartments involved in cross-presentation. As endosomes and phagosomes mature, the pH drops and the level of proteolysis rises. Late phagosomes become incompetent for cross-presentation, but acquire the characteristics required for peptide loading on MHC class II molecules.

In the **vacuolar pathway**, antigens are internalized into early phagosomes where they can be degraded into oligopeptides by endosomal proteases such as cathepsin S. The resulting peptides are loaded onto MHC class I molecules that are present in these compartments.

In the **phagosome-to-cytosol pathway**, antigens are internalized in the same early compartments and then are transferred to the cytosol maybe through Sec61 or an other mechanism. After proteasome processing in the cytosol, the resulting peptides can either be translocated into the ER via TAP and follow the endogenous route for MHC class I presentation, or be re-imported into early phagosomes via TAP for loading on MHC class I molecules (adapted from ref. 311).

Interestingly, loss of ERAP1 decreases the presentation of some epitopes from full-length proteins, does not affect others and yet, increases the presentation of others. Consequently, ERAP1 deficiency markedly changes CD8⁺ T cell responses to various epitopes in vivo and alters immunodominance hierarchies; reviewed in (309).

In contrast to ERAP1, ERAP2 does not stop trimming peptides when they are the size of mature epitopes (306). Although ERAP2 was shown to contribute to the processing of one epitope, its contribution to overall trimming is not yet clear (301). Silencing of ERAP2 only reduced overall MHC class I molecule expression by ~10%. Since this enzyme is not present in mice and poorly expressed in several human tissues, it seems to play a more limited or specialized role in peptide trimming.

6. Cross-presentation of antigenic peptides

Certain antigen-presenting cells, such as dendritic cells (DC), have the unique ability to present at their surface MHC class I molecules in complex with peptides that are derived from exogenous antigens through a process called cross-presentation. After acquiring these antigens in the periphery, DCs migrate to secondary lymphoid tissues where they can present these peptides to naive CD8⁺ T cells to initiate immune responses. This phenomenon is called cross-priming. The ability to present exogenous antigens plays a critical role in immune surveillance by allowing the immune system to monitor tissues for the presence of cancer cells, as well as viruses that do not infect DCs. Antigens are internalized in phagosomes or endosomes of DCs by different mechanisms: phagocytosis for particulate antigens and receptor-mediated endocytosis or pinocytosis for soluble antigens; reviewed in (310). Although the intracellular compartments and the molecular mechanisms involved in cross-presentation are still not completely defined, two major pathways by which cross-presented peptides are generated were characterized. These are the phagosome-to-cytosol pathway, which is TAP-dependent and proteasome-dependent, and the vacuolar pathway, which is presumably independent of any cytosolic step; reviewed in (311, 312) (**Figure 17**).

In the **phagosome-to-cytosol pathway**, cross-presented peptides are not directly generated in the phagosomes but are processed in the cytosol, where proteasomes are thought to make the initial cleavages on the parent protein (313). This pathway also appears to be dependent on TAP transporter, and sometimes requires ERAP1 activity (314, 315). Thus, once the exogenous antigen has reached the cytosol, it is thought to be processed for cross-presentation in the same

manner as endogenous proteins, being cleaved by proteasomes and transported by TAP into the ER. In addition, several studies provided evidence for the presence of many ER components in phagosomes or endosomes, including MHC class I molecules and TAP (316-319). This suggests that some peptides processed in the cytosol might be transported by TAP to bind MHC class I molecules in phagosomes. However, even though the ER membrane was found to contribute to phagosome formation in one study (320), this observation was not confirmed in another (321). The involvement of the ER in phagosome formation or maturation is still a matter of debate and the molecular mechanisms that would control this potential interaction remains to be established. Other evidence supporting a role for endocytic compartments in peptide loading during this pathway came from the identification of an endosomal aminopeptidase, IRAP (for insulin-regulated aminopeptidase), whose deficiency in DCs impaired cross-presentation but did not affect endogenous presentation (322). Interestingly, IRAP is closely related to ERAP1 and ERAP2 and can remove most amino acids from the N terminus of peptides. The authors of this study also observed a reduction of cross-presentation in ERAP1-deficient DCs, which was comparable to that observed in IRAP-deficient cells. It is presently unknown whether these two peptidases are working together in the phagosome or in separate compartments (ERAP1 in the ER and IRAP in the phagosome).

Additionally, the phagosome-to-cytosol pathway implies a transfer of the internalized antigen from the endocytic compartment to the cytosol. The molecular machinery involved in this transfer remains poorly defined, although a role of the retrotranslocation complex components Sec61 β , Derlin-1 and p97 was described in cross-presentation (316). This would be consistent with the observation that phagosomes contain components of the ER membrane. However, these findings should be taken with a pinch of salt since the composition of the retrotranslocation complex itself remains a matter of debate (§ I-6).

In the **vacuolar pathway** of cross-presentation, internalized antigens are processed in an endosome or a phagosome and the resulting peptides are directly loaded onto MHC class I molecules in the same endocytic compartment. This presentation, which is proteasome- and TAP-independent, seems to follow the same endosomal pathway as for MHC class II antigenic peptides. However, a distinction has to be made between early endocytic compartments, which possess neutral pH and low lysosomal peptidase activity, and late endocytic compartments, in which the pH drops and proteases are more active. Due to these properties, early phagosomes in DCs appeared to be the endocytic compartments involved in cross-presentation (323). Recent work reported that antigens delivered to late endocytic compartments were exclusively presented on class II molecules, whereas antigens delivered to early endosomes were presented on both

classes of MHC molecules (324). This could be explained by the fact that MHC class I molecules may not stably bind peptides at acidic pH.

In the vacuolar pathway, the production of certain cross-presented peptides was inhibited by cysteine protease inhibitors such as leupeptin (325). One of the vacuolar cysteine proteases identified for this process is cathepsin S. Indeed, cathepsin S-deficient mice and their DCs showed a defect in vacuolar but not in phagosome-to-cytosol cross-presentation. This was not the case for mice that were deficient for other cathepsins. These observations, together with the involvement of early endosomes in cross-presentation, are consistent with the fact that cathepsin S is relatively unique in being strongly active at neutral pH (326). However, additional endosomal proteases may also play a role in this pathway since a vacuolar cross-presented epitope was found to be efficiently processed after inhibition of cathepsins (324). Furthermore, IRAP might also be involved in the trimming of peptides during the vacuolar pathway of cross-presentation (322).

III) Protein splicing

Protein splicing is a rare post-translational processing event in which an internal protein segment is removed from a precursor protein with the concomitant ligation of the flanking fragments. Two principal types of protein splicing can be distinguished: an autocatalytic splicing and a protease-catalysed splicing. In autocatalytic splicing, the protein segment which is removed is termed “intein” and can be considered as an autocatalytic enzyme-like protein. No external energy or protein cofactor is required for this process, which is dependent, instead, on specific catalytic amino acid residues and involves intramolecular bond rearrangements. On the contrary, protease-catalysed splicing requires cleavage of peptide bonds by an enzyme and creation of a new peptide bond between released fragments. Although proteases are the principal effectors in protein degradation, mechanistically nothing prevents them from catalysing the “reverse” of the hydrolysis reaction, which is peptide bond synthesis. However, this phenomenon is not favored under physiological conditions and only a few examples of protease-catalysed splicing are described in nature. Indeed, even though several proteases were found to be able to catalyse peptide bond formation *in vitro* (which could be enhanced in the presence of organic solvents), only two proteases were reported to catalyse splicing *in vivo* (327). In plants, asparaginyl endopeptidase (AEP) was found responsible for splicing events leading to the maturation of the

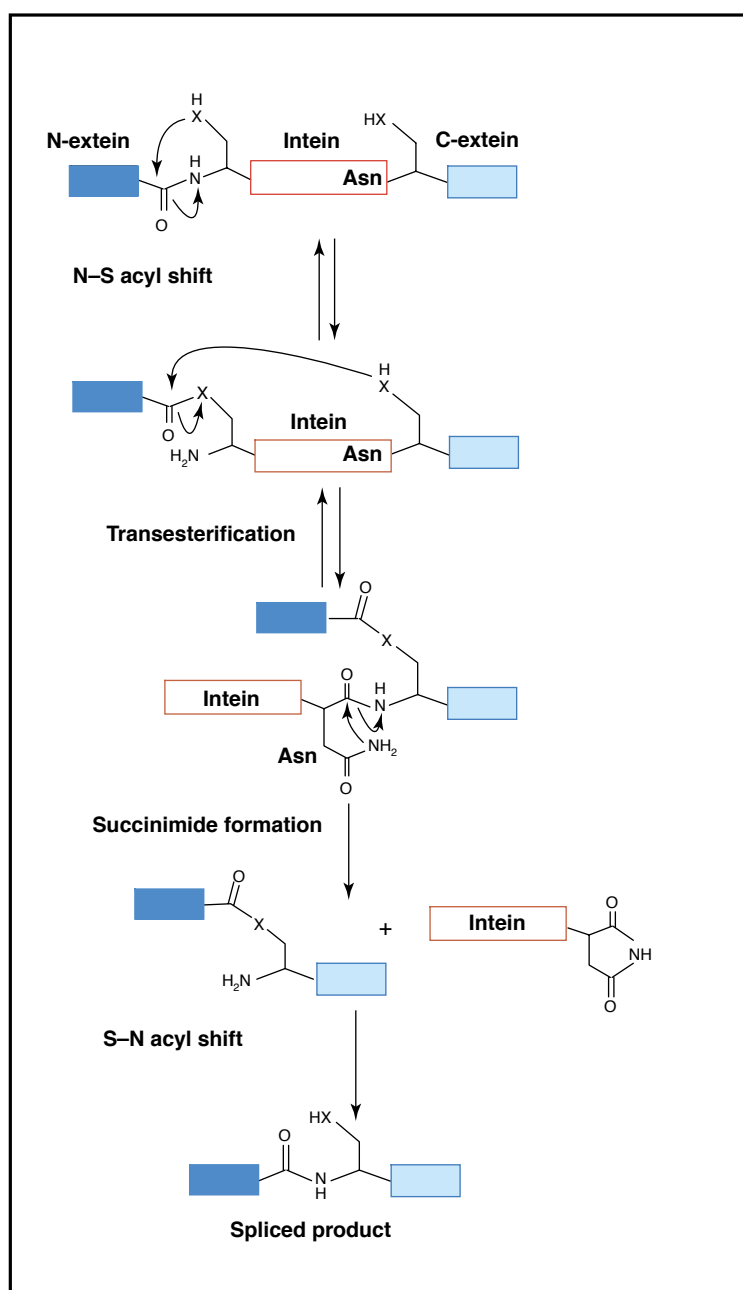


Figure 18. Mechanism of autocatalytic splicing. The hydroxyl- or sulfhydryl-containing side chain of the intron N-terminal residue initiates an N-O or N-S acyl rearrangement, resulting in an ester linkage between the N-extein and the intron. The side chain of cysteine, serine or threonine at the C-terminal splice junction attacks the ester in a transesterification reaction to form a branched intermediate. The intron is excised from the branch by cyclisation of the intron C-terminal asparagine coupled to peptide bond breakage. A spontaneous O-N or S-N shift generates a native peptide bond. The X represents either an oxygen or a sulfur atom (329).

lectin concanavalin A and to the biosynthesis of circular proteins known as cyclotides. In humans, the proteasome was shown to catalyse the production of spliced antigenic peptides presented onto MHC class I molecules.

In addition, it should be noted that potential protein splicing events were described in prokaryotes and eukaryotes, giving rise mostly to circular peptides or circular proteins (328). However, in most cases, the mechanisms involved remain to be elucidated.

1. Autocatalytic splicing

The process of autocatalytic splicing is described in many unicellular organisms prokaryotes and eukaryotes, as well as in viruses. This is an intramolecular process that relies on intervening protein sequences (inteins) capable of auto-excision and splicing of the remained external fragments (exteins). Since their discovery in 1990, more than 200 inteins have been identified, ranging from 128 to 1650 amino acids (329). Here, protein splicing is catalysed entirely by amino acid residues contained in the intein, so that no coenzymes or sources of metabolic energy are required. Conserved amino acids at the intein-extein borders (termed splice junctions) are observed (**Figure 18**). Most inteins start with a cysteine or a serine residue, which is responsible for an acyl-shift at the N-terminal splice junction. The first C-extein residue is invariably a cysteine, serine or threonine and the sulfhydryl or hydroxyl group of their side chain produces a nucleophilic attack on the linkage at the N-terminal splice junction, resulting in a branched intermediate. Another highly conserved group is the asparagine residue at the intein C terminus, which is involved in the resolution of the branched intermediate by side chain cyclisation. So, the steps that underlie autocatalytic splicing consist of two acyl-rearrangements, a transesterification and cyclization of an asparagine. Intein-based methodologies were developed to allow in vitro and in vivo protein processing in ways previously not possible using traditional biochemical and genetic approaches. Inteins are mainly utilized in the production of protein and peptide arrays, as molecular switches and in the reconstitution of functional proteins by split-gene techniques (329).

2. Splicing by asparaginyl endopeptidase in plants

Asparaginyl endopeptidases (AEP), also called legumains, are vacuolar cysteine proteases that specifically cleave peptide bonds at asparagine residues. They are widespread in plants and have

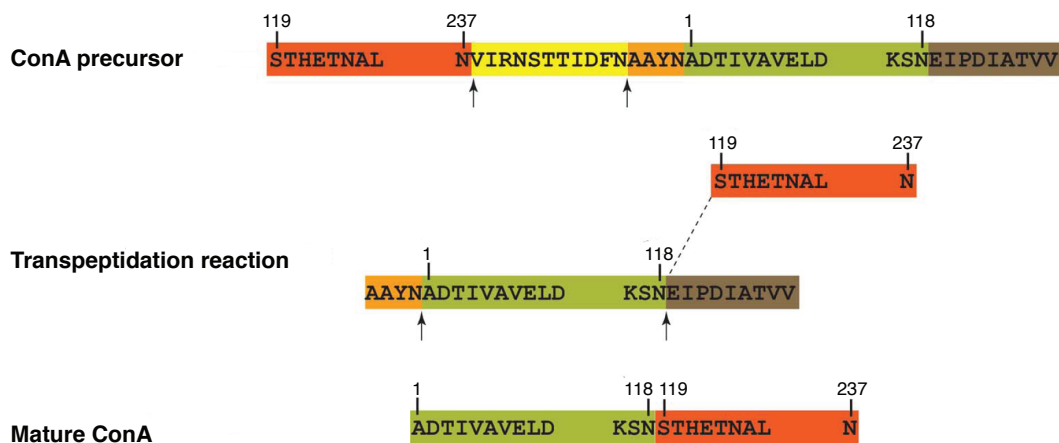


Figure 19. Model for concanavalin A (ConA) maturation involving asparaginyl endopeptidase (AEP).

The ConA precursor and intermediate peptides produced during maturation, with amino acids numbered according to their position in the mature ConA. AEP processing sites are indicated by arrows. Peptide bond formation occurs between Asn118 and Ser119 via a transpeptidation reaction (328).

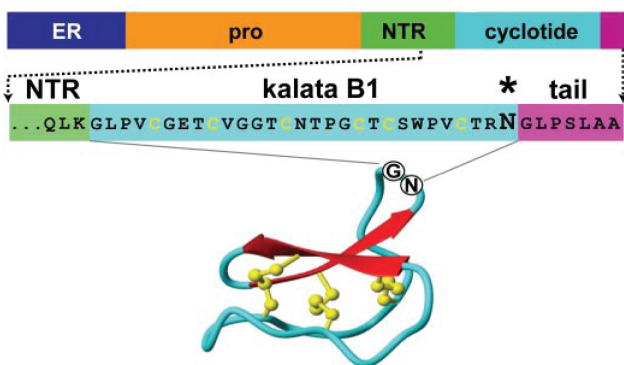


Figure 20. Cyclotide structure and precursor protein organization.

The structure of the cyclotide kalata B1 is shown in the ribbon representation with the Asn and Gly residues where cyclization takes place. Above is a schematic representation of the organization of the cyclotide precursors. The predicted ER signal sequence is followed by a pro-region, an N-terminal repeat region (NTR), the cyclotide domain and a short C-terminal tail region. The conserved Asn residue is marked with an asterisk and the Cys residues involved in the cystine knot are indicated in yellow (334).

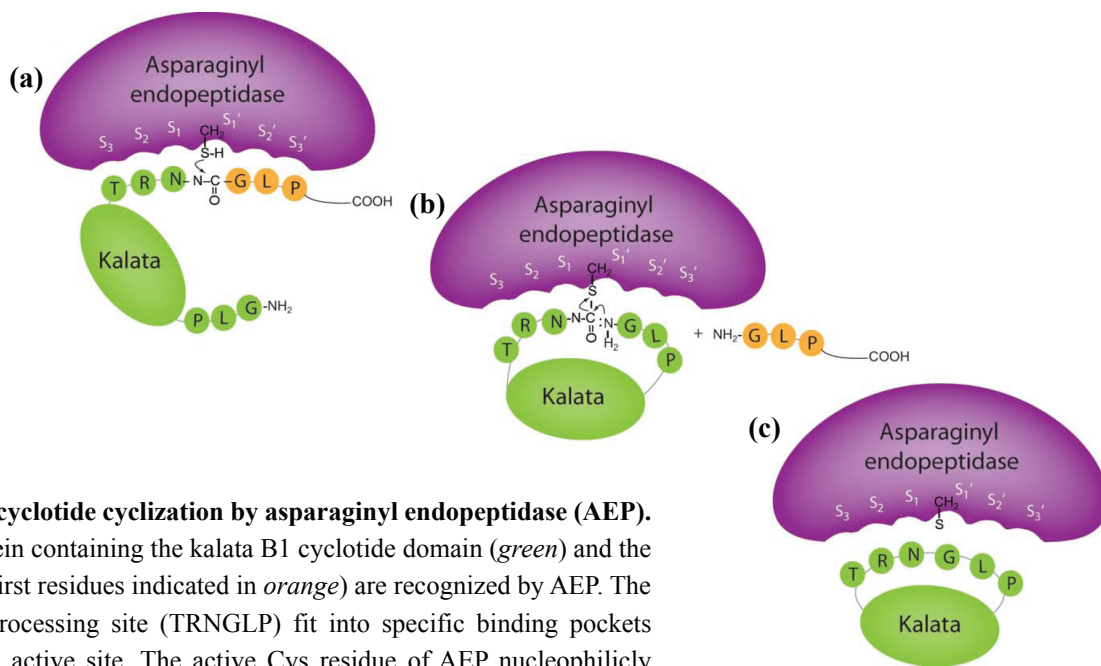


Figure 21. Model for cyclotide cyclization by asparaginyl endopeptidase (AEP).

(a) The precursor protein containing the kalata B1 cyclotide domain (green) and the C-terminal tail (three first residues indicated in orange) are recognized by AEP. The residues around the processing site (TRNGLP) fit into specific binding pockets present at the enzyme active site. The active Cys residue of AEP nucleophilically attacks the peptide bond after the Asn residue of kalata, releasing the C-terminal tail and forming an acyl-enzyme intermediate with the cyclotide domain.

(b) The N-terminal tripeptide residues of the cyclotide domain (GLP) fit correctly in the newly vacated primed substrate binding pockets (S'), allowing the amine group of N-terminal Gly residue to perform a nucleophilic attack on the acyl-enzyme intermediate. This leads to the formation of a new peptide bond and the cyclization of the cyclotide domain.

(c) The final cyclic peptide is then released from the enzyme active site (328).

a role in the activation and degradation of storage proteins, as well as in mediating programmed cell death in response to infection (330, 331). Interestingly, one AEP was found to be implicated in the catalysis of two distinct splicing events in plants.

2.1. *Maturation of concanavalin A*

The maturation of concanavalin A (ConA) leads to circular permutation of the protein sequence, a process in which the termini of the protein are linked by a peptide bond and new termini are created elsewhere in the protein by cleavage of an existing peptide bond (332). In the case of ConA, a 33.5 kDa precursor is cleaved into fragments that are re-ligated in the reverse order, yielding to the mature 30 kDa protein and the concomitant removal of a C-terminal peptide fragment (**Figure 19**). This post-translational splicing is catalysed by an AEP in a transpeptidation process presumably facilitated by a specific structural motif that favors a close proximity of the two fragments to be spliced together in the catalytic site of the enzyme. This proximity effect would explain why transpeptidation is favored over hydrolysis in this particular case (327). Furthermore, in this transpeptidation event, the energy released from peptide bond hydrolysis is utilized for subsequent peptide bond formation.

2.2. *Cyclization of cyclotides*

Two decades after an AEP was identified as a catalyst for peptide ligation in ConA maturation, AEPs were once again implicated in protein splicing in plants. The biosynthesis of cyclized proteins known as cyclotides was indeed recently found to involve an AEP-catalysed transpeptidation event (333, 334). The role of this process is of importance because non-cyclized analogues lack the bioactivity of their cyclized counterparts and the cyclized proteins display remarkably enhanced stability (332).

Cyclotides are characterized by a cystine knot of three disulfide bonds and a cyclic backbone (**Figure 20**). They are processed from precursor proteins that have both N- and C-terminal pro-regions. Mutation of a highly conserved asparagine residue at the C terminus of the cyclotide domain eliminates cyclization, as does deletion of the C-terminal pro-region following this residue. Cyclization was found to be dependent on AEP activity and the mechanism proposed involves the hydrolysis of the asparaginyl bond at the C terminus of the cyclotide domain, providing the energy to ligate the termini of the protein by transpeptidation (**Figure 21**). A close proximity of these two ends for the final cyclization event is presumably mediated either by the

structure of the cyclotide domain imposed by the cystine knot, or by the tripeptide motif present both at the N terminus and at the flanking C-terminal side of the cyclotide domain (amino acids G, L, P in the case of figures 20 and 21). The affinity of this tripeptide motif for the primed specific binding pockets of AEP is considered essential not only for the initial binding event, but also for the secondary binding event upon cleavage after the C-terminal asparagine of the cyclotide domain (328).

3. Splicing of antigenic peptides by the proteasome

Antigenic peptides presented by MHC class I molecules are generally made of a contiguous sequence of 8-11 amino acids cleaved from the parental protein. Recently, three antigenic peptides which were composed of two non-contiguous peptide fragments from a parent protein were identified, and constitute the first peptide splicing events described in vertebrates so far. These spliced peptides were recognized by specific CTLs isolated from the blood of cancer patients. Among the peptides identified, one is derived from fibroblast growth factor-5 (FGF-5), one from melanosomal protein gp100, and another is derived from nuclear protein SP110. The mechanism of production was studied for two of them and it was proposed that peptide splicing occurred within the proteasome by transpeptidation as described below.

3.1. Spliced antigenic peptides

In 2004, Hanada and coworkers were the first to identify a spliced antigenic peptide. This epitope, derived from fibroblast growth factor-5 (FGF-5), is presented by HLA-A3 molecules to a CTL clone that was isolated from human lymphocytes infiltrating a renal cell carcinoma (335). The minimal sequence of FGF-5 that was able to stimulate the CTL encoded 60 amino acids and none of the 8-mer to 10-mer peptides encoded within this sequence was able to stimulate the CTL. Internally deleted minigene constructs were further generated and tested. This led to the definition of a minimal stimulatory sequence made of two non-contiguous fragments of FGF-5. Next, an alanine scan was performed on this truncated, internally deleted sequence, by substituting each amino acid by an alanine (or a glycine in case of alanine). This allowed to the determination of which amino acids were crucial for the recognition by the CTL clone. Only the 1st, the 3rd, the 5th and the last four amino acids seemed to be important. By fusing the two non-contiguous peptide fragments that included all these crucial amino acids, the authors found that the resulting 9-mer peptide displayed appropriate HLA-A3 binding motifs and indeed strongly

FGF-5 :

MSLSFLLLLFFSHLILSAWAHGEKRLAPKGQPGPAATDRNPIGSSSRQSSSSA
MSSSSASSSPAASLGSGGLEQSSSFQWSPSGRRTGSLYCRVGIGFHLQIYPDGKVN
GSHEANMLSVLEIFAVSQGIVGIRGVFSNKFLAMSKKGLHASAKFTDDCKFRERFQ
ENSY**NTYAS**AIHRTEKTGREWYVALNKRKGAKRGCSPRVKPQHISTHFL**PRFK**QSE
QPELSFTVTVP**PEKKNPPSPIKSKIPLSAPRKNTNSVKYRLKFRFG**

NTYASPRFK

gp100 :

MDLVLRCLLHLAVIGALLAVGATKVPRNQDWLGVSQRL**RTK**AWNR**QLYPEW**TEAQ
RLDCWRGGQVSLKVSNDGPTLIGANASFSIALNFPQSQKVLDPGQVIWVNTIINGS
QVWGGQPVYPQETDDACIFPDGGPCPSGWSQKRSFVYVWKTWGQYWQVLGGPVSGL
SIGTGRAMLGTHTMEVTVYHRRGSRSYVPLAHSSSAFTITDQVPFSVSVSQLRALDG
GNKHFLRNQPLTFALQLHDPGSGYLAEADLSYTWDFGDSSGTLISRALVVTHTYLEPG
PVT AQVVLQAAIPLTSCGSSPVPGTTDGHRTAEAPNTTAGQVPTTEVVGTTPGQAP
TAEPSGTTSVQVPTTEVISTAPVQMPTAESTGMTPEKVPVSVVMGTTLAEMSTPEAT
GMTPAEVSIVVLSGTTAAQVTTTEWVETTARELPPIPEPEGPLASSIMSTESITGSLG
PLLDGTATLRLVKRQVPLDCVLRYGVSFVTLDIVQGIASAILQAVPSGEGDAFEL
TVSCQGGPLKEACMEISSPGCQPPAQRQCQVLPSPACQLVLHQILKGSGTYCLNV
SLADTNSLAVVSTQLIMPQGEAGLGQVPLIVGILLVLMAVVLASLIYRRLMKQDFS
VPQLPHSSSHWLRLPRIFCSCPIGENSPLLSGQQV

RTKQLYPEW

SP110 :

MFTMTRAMEEALFQHFHMQKLGIAIAIHKPFPFFEGLLDNSIITKRMYESLEACRN
LIPVSRVVHNILTQLERTFNLSLLVTLFSQINLREYPNLVTIYRSFKRVGASYEWQS
RDTPIILLEAPTGLAEGSSLHTPLALPHHKPPQPCSPCAPRVSEPGTSSQQSTEILS
ESPSPSDPVLPLPALIQEGRSTSVTNDKLTSMNAEEDSEEMPSLLTSTVQVASDNL
IPQIRDKEDPQEMPHSPLGSMPEIRDNSPEPNDPEEPQEVSSTPSDKKGKKRRCIW
STPKRRHKKK**SLPRGT**ASSRHGIQKKLKRVDQVPQKKDDSTCNSVETRAQKARTE
CARKSRSEEIIDGTSEMNEGKRSQKTPSTPRRVTQGAASPGHGIQEKLVVDKVTQR
KDDSTWNSEVMRVRQKARTKCARSKRLKEKKKEKDICSSSKRRFQKNIHRRGKPKSD
TVDFNCSKLPYTCGEAKGILYKKMKHGS SVK CIRNEDGTWLT PNEFEVEGKGRNAK
NWKRNI RCEBMTLGELLKRKNSDECEVCCGGQLLCCGTCPRVFHEDCHIPPVEAKR
MLWSCTEFCRMKRSSGSQCHVSKTLERQMOPQDQLIRDYGEFPQEAMWLDLVKERL
ITEMYTVAFVVRDMRLMFRNHKTFYKASDFGQVGLDLEAEFEKDLKDVLGFHEANDG
GFWTLF

SLPRGTSTPK

Figure 22. Spliced antigenic peptides.

Sequences of the parental protein and spliced antigenic peptide of FGF-5, melanocytic protein gp100 and nuclear protein SP110 (93).

stimulated the CTL. This peptide consists of fragments NTYAS and PRFK, which are separated by 40 residues in the original FGF-5 sequence (**Figure 22**). The identity of the natural antigenic peptide was finally confirmed using HPLC, by showing that the epitope presented on the surface of FGF-5-expressing cells and extracted by acid elution, co-eluted on the column with the synthetic 9-mer NTYASPRFK. Even though the mechanism of production of this spliced peptide was not studied here, a role of the proteasome was proposed. Indeed, the presentation of this epitope was impaired by treatment of autologous cells with proteasome inhibitor lactacystin.

The second spliced antigenic peptide was identified the same year in our laboratory (336). A clone of CD8⁺ lymphocytes was isolated from a melanoma patient and recognized an antigenic peptide presented by HLA-A32 and derived from the melanosomal glycoprotein gp100. The sequence of the epitope was identified using a similar approach and was included in a 13-residue minimal region. The antigenic peptide results from the fusion of two fragments, RTK and QLYPEW, which are separated by four amino acids in the gp100 sequence (**Figure 22**). The role of the proteasome in the production of this spliced peptide was determined by incubating a synthetic precursor peptide with purified 20S proteasomes. The proteasome was indeed able to produce the epitope in vitro, presumably by cleaving the precursor peptide and catalysing the formation of a new peptide bond between the two distant fragments. Interestingly, this production did not require any exogenous energy, suggesting that the energy necessary to form the new peptide bond might be recovered from one of the peptide bonds cleaved by the proteasome. This was confirmed by another set of in vitro experiments that were used to validate the proposed model of peptide splicing by transpeptidation (see below).

The third spliced antigenic peptide was discovered two years later and is a minor histocompatibility antigen created by a polymorphism in the *SP110* gene (337). This peptide is presented by HLA-A3 molecules and is recognized by CTLs isolated from a myeloma patient after MHC-matched hematopoietic cell transplantation. It is made of two non-contiguous fragments of four and six residues, STPK and SLPRGT, that are separated by six amino acids in the sequence of SP110 protein (**Figure 22**). In this case, these two distant fragments are spliced together in the reverse order to that in which they appear in the parental protein, to form the reordered spliced peptide SLPRGTSTPK. The mechanism of production was studied and the proteasome appeared responsible for the splicing reaction. The proposed model is also a transpeptidation, which allows the reordering of the peptide fragments before their ligation.

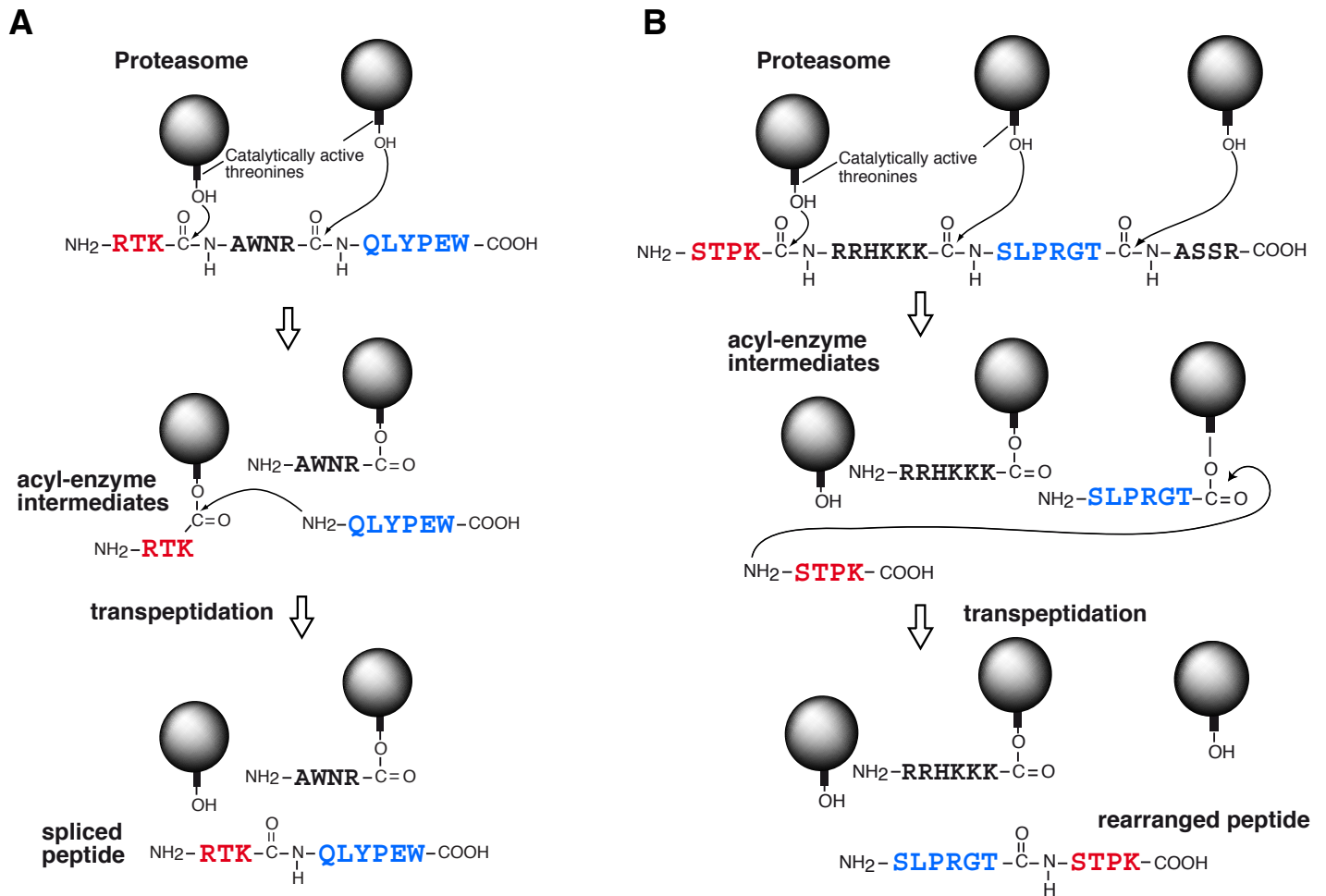


Figure 23. Transpeptidation model of peptide splicing inside the proteasome.

The balls represent the catalytically active β -subunits of the proteasome with the hydroxyl group of the side chain of the N-terminal threonine. Model for the splicing reaction leading to the gp100 spliced antigenic peptide (A) and to the rearranged spliced peptide derived from SP110 (B).

3.2 Transpeptidation model of peptide splicing by the proteasome

The gp100 and SP110 spliced peptides were shown to be produced by transpeptidation inside the catalytic chamber of the proteasome (336, 337) (**Figure 23**).

During peptide bond cleavage, the hydroxyl group of the N-terminal threonine of a catalytic β -subunit produces a nucleophilic attack on the peptide bond, leading to the production of a peptide with a free N terminus and to the formation of an acyl-enzyme intermediate between the N-terminal peptide fragment and the proteasome (§ Fig. 6). Normally, the acyl-enzyme intermediate is rapidly hydrolysed by a water molecule present at the active site. However, in the confined catalytic chamber of the proteasome, the acyl-enzyme intermediate is surrounded by a number of peptide fragments, whose N-terminal amino group can compete with water molecules and occasionally produce a nucleophilic attack on the intermediate, thereby creating a new spliced peptide by transpeptidation. In both cases, this model was validated by incubating various peptides combined in a pairwise manner together with purified proteasomes, and by testing the resulting digests for their ability to stimulate the CTL (336, 337). The energy required to create the new peptide bond of the spliced peptide must be recovered from one of the bonds cleaved by the proteasome since a proteasomal digest of the final fragments of the spliced peptide, which would not need additional cleavage, failed to produce the antigenic peptide. Transpeptidation is presumably favored within the catalytic chamber of the proteasome because of its confined space, which prevents rapid diffusion of the peptide fragments and increases their relative concentration. However, the efficiency of peptide splicing by the proteasome seems to be very low. Indeed, the comparison of the level of CTL activation obtained with the digests or with a titration curve of the corresponding synthetic peptide identified one spliced peptide produced from about 10^4 molecules of precursor peptides (336). It should be noted, however, that this observation only concerns in vitro digestions and does not provide any information on the efficiency of splicing inside the cell. In addition, no particular motif in the peptide sequence seems to be required for peptide splicing inside the proteasome, presumably due to the broad specificity of proteasomal cleavages. However, cleavage of a given peptide bond and concomitant acyl-enzyme formation can only occur if the original peptide has an amino acid composition suitable for efficient stabilisation at both the non-primed and the primed substrate-binding sites (§ I-2.3), which therefore may influence the nature of the peptide fragments to be spliced.

Objectives of the work

The aim of this work was to continue the recent findings on peptide splicing by the proteasome to gain more insight on its mechanism and importance. The study can be divided into three parts, each representing a publication.

The mechanism of production of peptide splicing was elucidated for two of the three spliced antigenic peptides described. However, the splicing mechanism was unknown for the spliced peptide derived from FGF-5. In this case, the much greater length of the intervening segment (40 aa) made the transpeptidation model more difficult to envisage. The first part of the work therefore consisted of studying the mechanism of production of the FGF-5 spliced peptide. This led us to evaluate the effect of the length of the intervening segment on the efficiency of splicing, but also to test the possibility of *trans*-splicing, which corresponds to the splicing of fragments coming from two distinct substrates.

In a second article, a comparative analysis of production of the three spliced peptides between the standard proteasome and the immunoproteasome was performed. Differential isotope labelling was used to quantify by mass spectrometry the fragments produced in digests obtained with precursor peptides and purified proteasomes. This technique allowed a precise comparison of the fragments produced by both proteasome types and provided information to explain the differential processing observed for each spliced peptide.

Finally, the third part of this work consisted of the identification of a new spliced antigenic peptide, which contains two deamidated aspartate residues. This peptide is derived from tyrosinase and is presented by HLA-A24 molecules to CTLs isolated from a melanoma patient. This work has been initiated by Dr. Paul Robbins from the NIH in Bethesda.

RESULTS

I) ARTICLE 1

SPLICING OF DISTANT PEPTIDE FRAGMENTS OCCURS IN THE PROTEASOME BY TRANSPEPTIDATION AND PRODUCES THE SPLICED ANTIGENIC PEPTIDE DERIVED FROM FIBROBLAST GROWTH FACTOR-5

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Benoît J. Van den Eynde*

Running title: Splicing of the FGF-5 antigenic peptide by the proteasome

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ABSTRACT

Peptide splicing is a newly described mode of production of antigenic peptides presented by MHC class I molecules, whereby two non-contiguous fragments of the parental protein are joined together after excision of the intervening segment. Three spliced peptides have been described. In two cases, splicing involved the excision of a short intervening segment of 4 or 6 amino acids, and was shown to occur in the proteasome by transpeptidation resulting from the nucleophilic attack of an acyl-enzyme intermediate by the N terminus of the other peptide fragment. For the third peptide, which is derived from fibroblast growth factor-5 (FGF-5), the splicing mechanism remains unknown. In this case, the intervening segment is 40 amino-acid long. This much greater length made the transpeptidation model more difficult to envision. We therefore evaluated the role of the proteasome in the splicing of this peptide. We observed that the spliced FGF-5 peptide was produced *in vitro* after incubation of proteasomes with a 49-amino acid long precursor peptide. We evaluated the catalytic mechanism by incubating proteasomes with various precursor peptides. The results confirmed the transpeptidation model of splicing. By transfecting a series of mutant FGF-5 constructs, we observed that reducing the length of the intervening segment increased the production of the spliced peptide, as predicted by the transpeptidation model. Finally, we observed that *trans*-splicing — i.e. splicing of fragments from two distinct proteins — can occur in the cell, but with a much lower efficacy than splicing of fragments from the same protein.

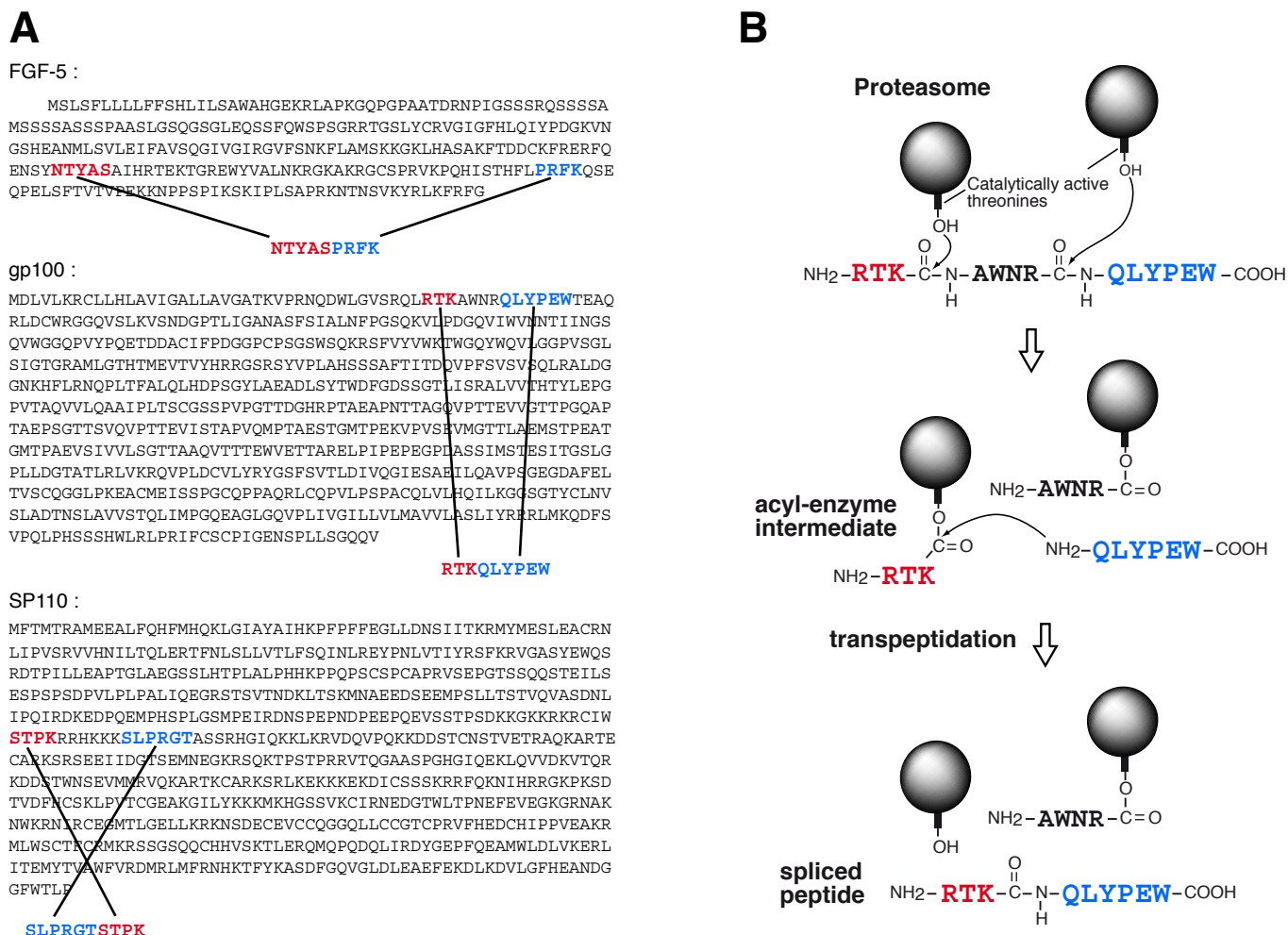


Figure 1. Spliced antigenic peptides.

(A) Sequences of the parental protein and spliced antigenic peptide of fibroblast growth factor-5 (FGF-5), melanocytic protein gp100 and nuclear protein SP110.

(B) Mechanism of the splicing reaction inside the proteasome. The balls represent the catalytically active β -subunits of the proteasome with the hydroxyl group of the side chain of the N-terminal threonine.

INTRODUCTION

Antigens presented by MHC class I molecules are composed of peptides of 8-11 amino acids in length and are recognized at the cell surface by specific CD8⁺ T lymphocytes. The presentation of these antigenic peptides by infected cells or tumor cells allows their specific recognition and lysis by T lymphocytes (1). These peptides are usually derived from the degradation of intracellular proteins by the proteasome. The 26S proteasome consists of a 20S core cylindrical complex capped at both ends by 19S regulatory particles (2). Catalytic activity is exerted by the 20S proteasome composed of four stacked rings of seven α - or β -subunits each (3). 19S regulatory complexes bind the α -rings of the 20S core and are responsible for substrate capture and translocation of these substrates into the 20S catalytic complex. Enzymatic activities are mediated by three of the seven β -subunits of the 20S proteasome: β 1, β 2, and β 5, corresponding respectively to three major activities: caspase-like, trypsin-like and chymotrypsin-like activities (4, 5). The 20S proteasome is classified as a N-terminal nucleophile hydrolase, with the amino-terminal threonine of the active β -subunits acting as the nucleophile catalyst (3, 6, 7).

Most MHC class I-associated antigenic peptides correspond to small continuous fragments of intracellular proteins. However, in the past few years, some antigenic peptides were found to be created by the splicing of two noncontiguous fragments of the respective parental proteins (Fig. 1A). The first example is a nine-amino acid peptide derived from fibroblast growth factor-5 (FGF-5) and made of two fragments of five and four residues which are located forty amino acids apart in the parental protein (8). Another is a nonameric peptide produced from the melanosomal protein gp100 by excision of four amino acids and splicing of the two flanking fragments, one of three and one of six residues (9). The third example of spliced peptide is a human minor histocompatibility antigen created by a polymorphism in the *SP110* gene (10). This epitope is made of two noncontiguous fragments of four and six residues, separated by six amino acids in the SP110 protein. In this case, the two fragments are spliced together but in the reverse order to that in which they appear in the parental protein.

The splicing mechanism was studied in detail for the gp100 and SP110 peptides (9, 10). In both cases, quite surprisingly, the proteasome was found to produce the spliced peptide, and the splicing reaction could be reproduced in vitro by incubating precursor peptides with purified proteasomes. The splicing process involves a first step where the intervening fragment is cleaved off. The amino-terminal fragment of the final epitope is retained on the catalytic subunit as an acyl-enzyme intermediate through an ester bond with the side chain of the catalytically active

threonine of one of the β -subunits (Fig. 1B). This ester bond then undergoes a nucleophilic attack by the free N-terminus of the other peptide fragment, which results in a transpeptidation reaction forming a new peptide bond. In this reaction, the peptide fragment competes for this nucleophilic attack with water molecules, which, during normal proteolysis, hydrolyze the ester bond of the acyl-enzyme intermediate. Transpeptidation is presumably favored within the catalytic chamber of the proteasome due to its confined space, which prevents rapid diffusion of the peptide fragments and increases their relative concentration (9, 11).

In the case of the FGF-5 spliced peptide, the mechanism of splicing has not been worked out. Hanada *et al.* showed that recognition of tumor cells by the cognate CTL was reduced after treatment with proteasome inhibitor lactacystin or after TAP inhibition with ICP47 (8). These results suggested that the proteasome was also involved in the production of this spliced peptide. However, the length of the intervening fragment to remove before splicing of the two flanking fragments, forty amino acids, made the transpeptidation model inside the proteasome more difficult to envision in this case, as it implies that the two distinct fragments remain within the catalytic chamber of the proteasome during the time needed to cleave off the long intervening segment. We therefore set out to confirm the role of the proteasome in the splicing of the FGF-5 peptide. This also led us to evaluate the influence of the length of the intervening segment on the efficiency of splicing, and to test the possibility of *trans*-splicing between two distinct proteins.

MATERIALS and METHODS

Cell lines and CTL clones

Autologous renal cell carcinoma line 1764-RCC and COS-7 cells were cultured in D-MEM medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). HEK293-EBNA cell line and EBV-transformed B cells were cultured in IMDM medium (Invitrogen) containing 10% FCS. All culture media were supplemented with L-arginine (116 mg/ml), L-asparagine (36 mg/ml); L-glutamine (216 mg/ml), penicillin (100 U/ml) and streptomycin (100 μ g/ml).

CTL C2 is a human CTL clone that recognizes the FGF-5 spliced peptide NTYASPRFK presented by HLA-A3 molecules (8). CTL clone 14 (full name CTL 35-7-14) recognizes spliced peptide gp100_{40-42/47-52} RTKQLYPEW associated with HLA-A32 (9). CTL clone 7 (full name CTL 606 C2/1) recognizes peptide gp100₂₀₉₋₂₁₇ ITDQVPFSV presented by HLA-A2 (12). These CTLs were expanded as previously described (13) by stimulation every 14 days with 40 ng/ml anti-CD3 OKT3 monoclonal antibody (Ortho Biotech Products), using irradiated (100 Gy) allogeneic PBMC, in IMDM medium containing 10% human serum, human rIL-2 (50 UI/ml), IDO inhibitor 1-methyl-L-tryptophan 200 μ M (Sigma-Aldrich) (14), and supplemented with amino acids and antibiotics as mentioned above.

Proteasome inhibition in autologous renal carcinoma cells

Cells were first treated at pH 3 for 30 seconds in a glycine buffer to elute MHC-bound peptides and neutralized with culture medium. Then, cells were treated for 1 hour at 37°C with 50 nM epoxomicin (Sigma-Aldrich) or with 20 μ M lactacystin (Bio Connect) in X-vivo 10 medium (BioWhittaker). Cells were washed with the same medium and then incubated for 4 hours with lower concentrations of inhibitors (10 nM epoxomicin or 2 μ M lactacystin) in a 96-well plate (30,000 cells/well in 100 μ l of medium). Supernatants were then carefully removed and half of the cells were loaded for 1 hour with the antigenic peptide (2 μ g/ml). Supernatants were removed just before the addition of 20,000 CTL C2 per well in 100 μ l of medium. The production of IFN γ was measured by ELISA (Biosource) after an overnight incubation.

Peptide electroporation followed by CTL assay

In Fig. 2B, HEK293-EBNA cells were treated for 1 hour with the indicated amounts of lactacystin. Cells were then washed with PBS and electroporated in 400 μ l electroporation buffer (K_2HPO_4/KH_2PO_4 10 mM pH 7.5, $MgCl_2$ 1 mM, sucrose 250 mM) with 100 μ M of 30-mer peptide NTYASAIHKRGCSPRVKPQHISTHFLPRFK in 0.4 cm cuvettes, using a Genepulser Xcell electroporator (Biorad), functioning in square wave mode with 10 pulses of 0.1 ms at 480V separated by 1 sec intervals. Cells were diluted to a peptide concentration of 6.7 μ M and incubated during 4 hours in culture medium containing or not 1 μ M lactacystin. Cells were washed and then half of them were loaded with antigenic peptide (2 μ g/ml) for 1 hour. For the CTL assay, 25,000 cells per well (96-well plate) were incubated overnight with 20,000 CTL C2. In Fig. 6B, COS-7 stably transfected with HLA-A3 were electroporated with 50 μ M of each peptide using the same parameters as above. Cells were then diluted to a peptide concentration of 3.3 μ M and incubated during 4 hours. After washing, half of the cells were loaded with the antigenic peptide (2 μ g/ml) during 30 min. For the CTL assay, 40,000 electroporated cells per well were incubated with 20,000 cells of CTL C2. In Fig. 7B, COS-7 cells stably transfected with HLA-A32 were electroporated with 70 μ M of each peptide. Cells were then diluted to a peptide concentration of 4.7 μ M and incubated during 4 hours. After washing, half of the cells were loaded with the antigenic peptide (2 μ g/ml) during 30 min. For the CTL assay, 40,000 electroporated cells per well were incubated with 25,000 cells of CTL clone 14 (9). The production of IFN γ was measured by ELISA after an overnight incubation.

Proteasome digestions of synthetic peptides and CTL assay of the digests

Synthetic peptides were produced using Fmoc solid phase chemistry. Peptides were dissolved in dimethyl sulfoxide at 20 mg/ml and stored at -20°C. 20S standard proteasomes were purified from human erythrocytes as previously described (15). Digestion of the FGF-5 49-mer precursor peptide (Fig. 3A) was performed by incubating at 37°C 8 μ g of peptide with 1 μ g of 20S proteasomes in 20 μ l of Tris 10 mM pH 7.5. In Fig. 4B, 4 μ g of each of the combined peptides was mixed with 1 μ g of 20S proteasomes in 20 μ l of Tris 10 mM pH 8. In Fig. 6A, we incubated 7 μ g of each precursor peptide with 1 μ g of 20S proteasomes in Tris 10mM pH 8. In Fig. 7A, 2 μ g of each precursor peptide were incubated with 1 μ g of 20S proteasomes in the same buffer. Digestions were stopped by addition of 2 μ l of 10% trifluoroacetic acid (TFA), lyophilized and

resuspended in 20 μ l of water. One half (Fig. 6A) or one quarter (Fig. 7A) of each digest was diluted in 100 μ l of X-vivo 10 medium and pulsed in duplicate wells containing 20,000 EBV-B cells expressing the appropriate HLA molecule. CTLs (20,000/well) were added after 30 min of incubation and the production of IFN γ in supernatants was measured by ELISA after an overnight co-culture.

Generation and analysis of mutated plasmid constructs

FGF-5 constructs with internal deletions were generated from a plasmid encoding the full length *FGF-5* gene by carrying out a long PCR with one primer, using QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene). For each deletion construct, we designed a primer composed by at least 20 nucleotides of the FGF-5 sequence situated on each side of the deletion. To generate the FGF-5 Δ 1 construct, encoding a deletion of amino acids 192 to 201, we used the primer

5'-GGGCGGGAGTGGTATGTGGCCTGCAGCCCCCGGGTTAAACCC-3'. For the FGF-5 Δ 2 construct, encoding deletion of amino acids 187 to 206, we used the primer

5'-AGAACTGAAAAACAGGGCGGAAACCCCAGCATATCTCTACCC-3' and for FGF-5 Δ 3, encoding deletion of amino acids 181 to 212, we used the primer

5'-CCTATGCCTCAGCAATACATAGAACCCATTTTCTGCCAAGATTCAAGC-3'. Then, we amplified, by PCR using a Taq polymerase, the entire FGF-5 coding region containing the internal deletion and cloned the PCR products into pEF6/V5-His TOPO (Invitrogen). Point mutations in the FGF-5 and gp100 sequences were also introduced by carrying out PCR with QuickChange® Multi Site-Directed Mutagenesis Kit. We used a pcDNA3 plasmid containing the wild-type *gp100* cDNA as template, and specific primers for each mutation required. For FGF-5, the template was plasmid pEF6 containing the full-length FGF-5 cDNA. The sequences of the coding region of all the plasmids generated were verified by sequencing with an ABI3130 Genetic Analyzer (Applied Biosystem).

Transient transfection followed by CTL assay

HEK293-EBNA cells (25,000 cells/well), which are HLA-A3⁺, were plated in flat-bottom 96-well microplate 48 hours before transfection. Cells were transfected using Lipofectamine reagent (Invitrogen) with the indicated amounts of pEF6 plasmids coding for the *FGF-5* genes described

above. Twenty-four hours after transfection, the medium was carefully removed and 25,000 cells of CTL C2 were added in X-vivo 10 medium. After an overnight incubation, the supernatants were collected and their content in IFN γ was measured by ELISA.

COS-7 cells (15,000 cells/well) were plated in flat-bottom 96-well microplate 24 hours before transfection. Cells were transfected using Lipofectamine with 50 ng of plasmid DNA coding for the appropriate HLA molecule (HLA-A3, -A32 or -A2), and 200 ng of plasmid DNA of either *FGF-5* or *gp100* plasmid construct (empty vector was added when required to transfect an equal amount of DNA in all conditions). CTL C2 (Fig. 6C), CTL 14 or CTL 7 (Fig. 7C) were added 24 hours later in X-vivo 10 medium and the supernatant was collected after 20 hours of co-culture to estimate its content in IFN γ .

Transient transfection of FGF-5 followed by kinetics of intracellular clearance and western blots

HEK293-EBNA cells (3×10^6 cells/flask) were plated in T75 flasks 24 hours before transfection. Cells were transfected using TransIT-LT1 reagent (Mirus) with 20 μ g of pEF6 plasmids with the FGF-5 constructs described above. Twenty-four hours after transfection, cells were detached and treated with 100 μ g/ml of cycloheximide (Sigma-Aldrich) during the indicated periods of time. After treatment, cells were washed with PBS and dry pellets were frozen immediately. Cells were further lysed on ice in Cytobuster buffer (Novagen) supplemented with recombinant DNase I (50 μ g/ml) and protease inhibitor cocktail (Complete, Roche Applied Science). Each sample was sonicated during 10-15 sec and centrifuged at 20,000g during 25 minutes at 4°C. Protein concentration in supernatants was determined with the BCA Protein Assay (Thermo Scientific). Twenty-five micrograms of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with primary antibodies (anti-FGF-5 : sc-1362 purchased from Cell Signaling or anti- β -actin: AC-15 from Sigma-Aldrich) and horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology), and developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

HPLC and Mass Spectrometry (MS)

Mass spectrometry was performed on line with a LCQ Deca XP Plus ion-trap mass spectrometer equipped with an electrospray ionization source (ThermoFinnigan). Mass spectra were acquired in a mode that alternated single MS scans (m/z 200-2000) with MS/MS scans. Samples were

separated by reverse-phase chromatography on a PepMap C18 0.3/15 column (LC Packings) and eluted using a 55-min linear gradient of acetonitrile in water (5-50%) containing 0.05% TFA with a flow rate of 4 μ l/min obtained by splitting.

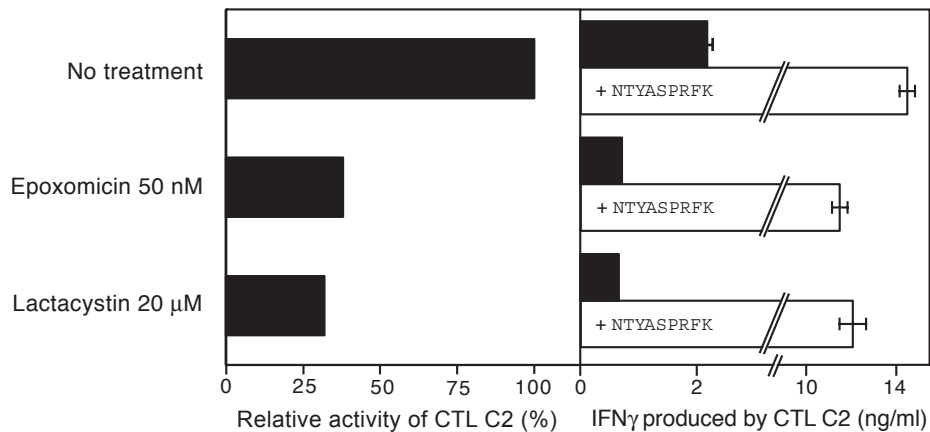
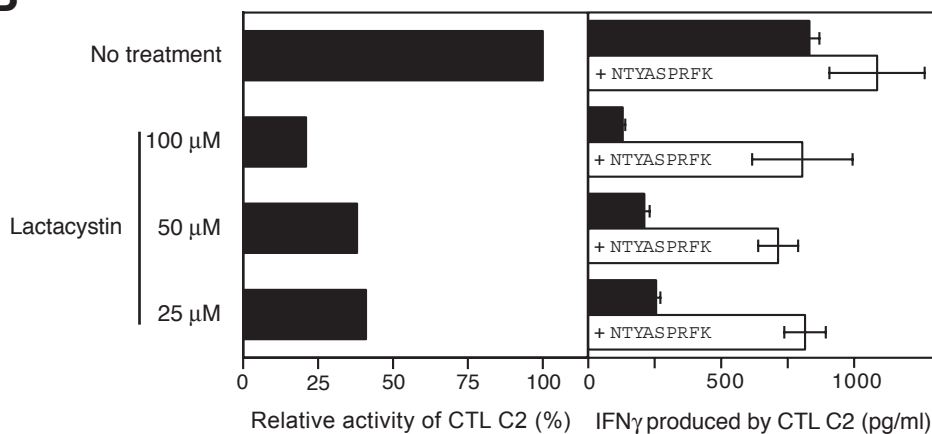
A**B**

Figure 2. Proteasome inhibition prevents the production of the spliced FGF-5 antigenic peptide.

(A) Autologous tumor cells 1764-RCC were treated with the indicated proteasome inhibitors after acid elution of MHC-bound peptides, and then tested for recognition by CTL clone C2. The production of interferon-gamma (IFN γ) was measured after an overnight incubation. Where indicated, cells were loaded with synthetic peptide NTYASPRFK before the CTL assay to check their antigen-presentation capacity. Relative activities (*left panel*) were calculated first as a proportion of the activity measured with the synthetic peptide control. This proportion was then related to that observed without treatment, which was taken as 100%. Actual values are shown on the right panel. Error bars show standard deviations of triplicates.

(B) Lactacystin-treated HEK293-EBNA cells were electroporated with a 30-mer precursor peptide (NTYASAIHKRGCSRVKPKQHITHFLPRFK) and tested for recognition by CTL C2. HEK293-EBNA cells naturally express HLA-A3. Where indicated, synthetic peptide NTYASPRFK was loaded during one hour before the CTL assay. The production of IFN γ was measured after an overnight incubation. Relative activities (*left panel*) were calculated as above. Actual values are shown on the right panel. Error bars show standard deviations of triplicates.

RESULTS

We first confirmed the involvement of the proteasome in the processing of the FGF-5 antigenic peptide by evaluating recognition of cells treated with proteasome inhibitor lactacystin or epoxomicin by FGF-5-specific CTL clone C2. We observed a reduced recognition of treated cells, not only with autologous tumor cells (Fig. 2A), but also with HEK293-EBNA cells electroporated with a precursor peptide encompassing the FGF-5 spliced peptide (Fig. 2B).

To directly evaluate the ability of the proteasome to produce the FGF-5 spliced peptide, we incubated purified 20S proteasomes with a 49-mer precursor peptide encompassing the complete sequence of FGF-5 between the two spliced fragments NTYAS and PRFK. We then examined recognition by CTL C2 of target cells loaded with the digests. The digests were clearly able to activate the CTL, and this ability increased with digestion time (Fig. 3A). The presence of the spliced antigenic peptide was evaluated by HPLC combined with tandem mass spectrometry (MS/MS) in a digest obtained after 240 min of incubation of the 49-mer precursor peptide with 20S proteasomes. The antigenic peptide was observed in this digest, and was detected as a doubly charged ion with m/z 542²⁺. Its retention time and fragmentation pattern were identical to those of the synthetic peptide NTYASPRFK (Fig. 3B).

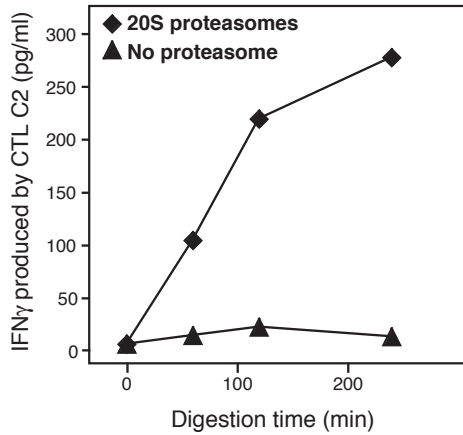
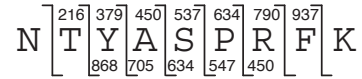
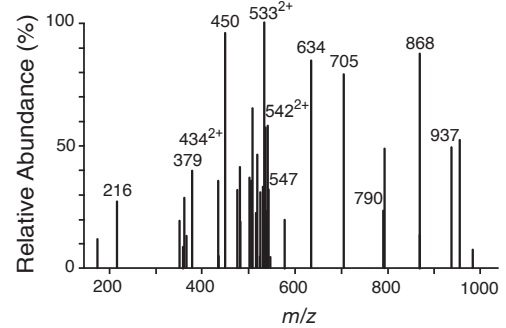
The efficiency of the splicing reaction was particularly low: a comparison of the level of CTL activation obtained with the digests or with a titration curve of the synthetic peptide provided a rough estimate of one spliced peptide produced from about 5×10^5 molecules of precursor peptide.

We also used mass spectrometry to estimate the degradation of the precursor peptide in those digestions. We observed a biphasic relation between production of the spliced peptide and precursor degradation, with a first linear phase likely corresponding to direct production of the spliced peptide from the long precursor, and a second phase when spliced peptide production kept increasing despite complete degradation of the precursor (Fig. S1A). The latter phase probably resulted from re-entry and further processing of degradation intermediates, a phenomenon favored by *in vitro* digestion conditions involving a single substrate. In line with this interpretation, we observed in those digests an identical biphasic production of the fragments to be spliced, NTYAS and PRFK (Fig. S1B).

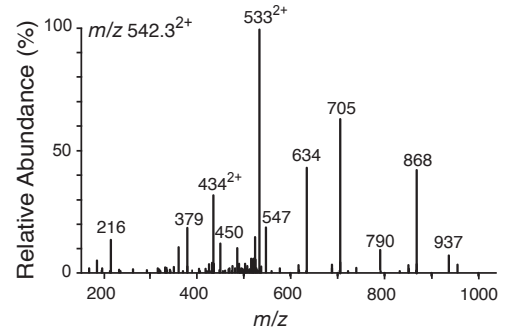
A

Precursor peptide :

NTYASAIHRTEKTGREWYVALNKR GKAKRGCSPRVKPQHISTHFLPRFK

**B** m/z 542.3²⁺ from 240min digest

Synthetic peptide NTYASPRFK

**Figure 3. In vitro production of the spliced FGF-5 antigenic peptide by proteasomes.**

(A) CTL recognition of digests obtained by in vitro incubation of the indicated FGF-5 49-mer precursor peptide with purified 20S standard proteasomes. Reaction was stopped at 0, 60, 120 and 240 minutes. Digests were then loaded onto HLA-A3⁺ EBV-B cells before addition of the CTL. Production of IFN γ was measured 18 hours later.

(B) MS/MS fragmentation spectrum of the doubly charged ion with m/z 542²⁺ observed in the 240-min digest shown in A, and MS/MS fragmentation spectrum of the doubly charged ion with m/z 542²⁺ of the synthetic peptide NTYAS-PRFK. The fragment ions that were detected are indicated above the peptide sequence for N-terminal b ions and below for C-terminal y ions. Ion m/z 533²⁺ is a dehydrated form of ion m/z 542²⁺.

If the FGF-5 peptide is produced in the proteasome by the same splicing mechanism as the two other spliced peptides, the fragment that would be involved in the acyl-enzyme intermediate would be NTYAS while fragment PRFK would be responsible for the nucleophilic attack of this intermediate to form the spliced peptide NTYASPRFK (Fig. 4A). The formation of the acyl-enzyme intermediate would be coupled to the cleavage of the peptide bond C-terminal from NTYAS in the parental polypeptide. We investigated the requirement for cleavage of this peptide bond by incubating 20S proteasomes with various synthetic peptides combined in a pairwise manner. Digests obtained with peptides NTYASAIH and PRFK were strongly recognized by the CTL, while digests obtained with peptides NTYAS and PRFK were not (Fig. 4B). This indicated that the formation of an acyl-enzyme intermediate linking NTYAS to the proteasome is required for the splicing reaction. This experiment also confirmed that the energy of the new peptide bond created is provided by the cleavage of the bond between residues S and A of fragment NTYASAIH. We then observed that production of the spliced peptide was abolished when the amino terminal group of fragment PRFK was blocked by acetylation (Fig. 4B). This result confirmed that this amino group was responsible for the nucleophilic attack of the acyl-enzyme intermediate, and thereby supports the transpeptidation model of splicing leading to the final antigenic peptide.

A major difference between the splicing of the FGF-5 peptide and the splicing of the gp100 and SP110 peptides studied before is the much longer length of the intervening sequence, forty amino acids instead of four and six. To evaluate the effect of the length of the intervening sequence, we constructed three plasmids encoding mutated FGF-5 with shortened intervening sequences of different lengths. These mutated FGF-5, called $\Delta 1$, $\Delta 2$ and $\Delta 3$, comprised an intervening sequence of respectively 30, 20 and 8 residues (Fig. 5A). Amino acids were deleted in the middle of the intervening sequence so as to maintain the residues flanking the two fragments of the final epitope and to minimize potential cleavage modifications at these sites. Increasing amounts of these plasmids were then transfected into HEK293-EBNA cells and the production of the spliced FGF-5 peptide was evaluated by measuring the ability of transfected cells to activate CTL C2. The efficiency of production of the spliced peptide clearly increased when the size of the intervening sequence was reduced (Fig. 5B). The highest CTL activation was observed with cells transfected with the construct encoding an intervening sequence of 8 amino acids, while the lower activation was observed with cells transfected with the wild-type construct encoding a 40-amino acid intervening segment. The result obtained with the mutant

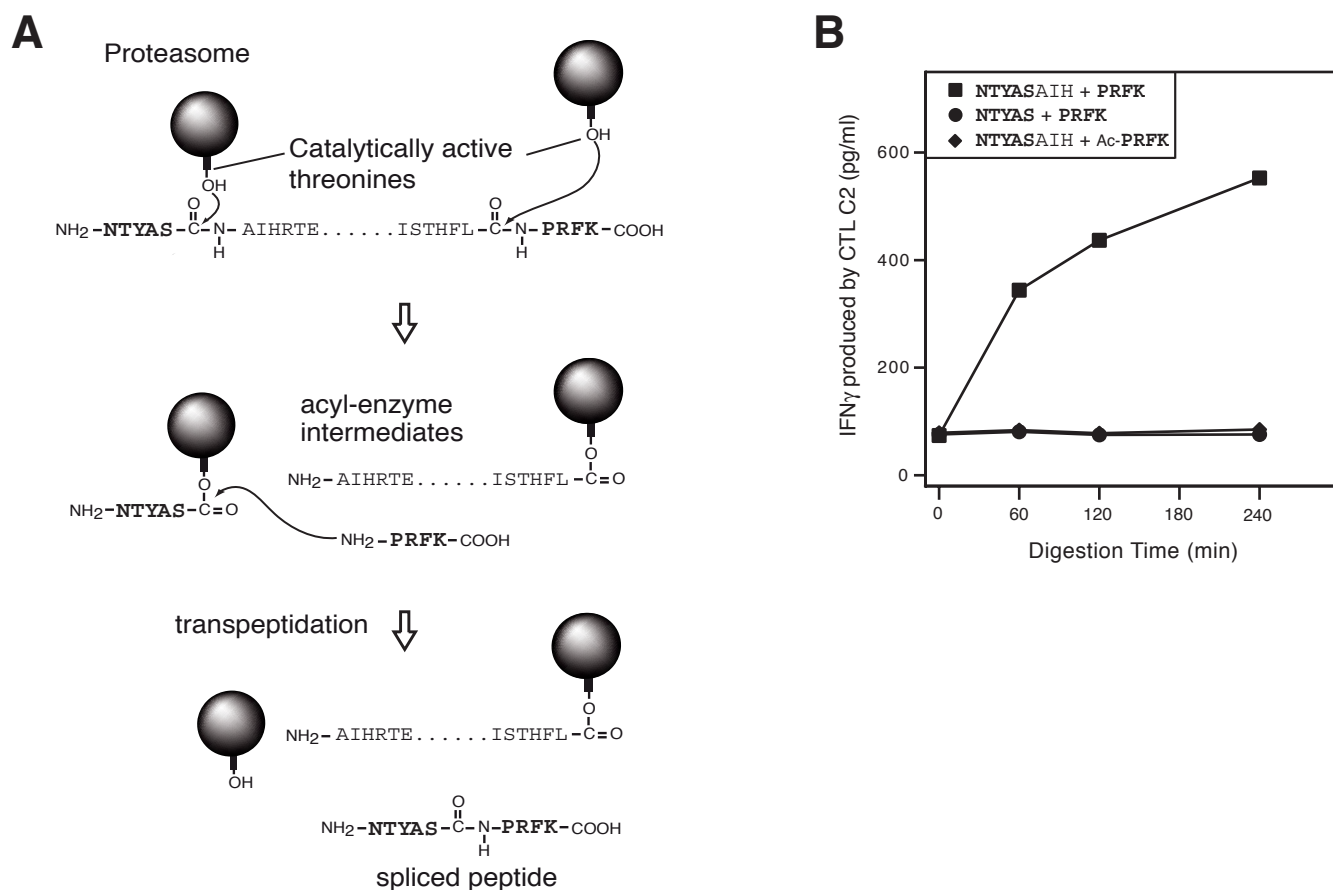


Figure 4. Mechanism of production of the spliced FGF-5 antigenic peptide.

(A) Model of the catalytic mechanism of the splicing reaction occurring within the proteasome to produce the FGF-5 spliced peptide. The balls represent the catalytically active β -subunits of the proteasome with the hydroxyl group of the side chain of the N-terminal threonine.

(B) CTL recognition of digests obtained by in vitro incubation of 20S proteasomes with various synthetic peptides combined in a pairwise manner. Digests obtained by incubating proteasomes with the indicated peptides were loaded onto HLA-A3+ EBV-B cells before addition of the CTL. Production of IFN γ was measured 18 hours later. Ac-PRFK: N-terminally acetylated peptide with sequence PRFK.

encoding the 30-amino acid segment was similar to that obtained with the wild-type. We considered that the better production of the peptide from the short mutants could result from a misfolding of the protein, which could increase its degradation by the proteasome. To exclude this possibility, we compared the degradation of the four different FGF-5 proteins in transfected cells. We performed western blots on transfected cells treated with cycloheximide, an inhibitor of protein synthesis, using an antibody directed against the C-terminus of FGF-5. This antibody detects three specific bands, presumably corresponding to three glycosylated forms of FGF-5 (Fig. 5C) (16). The proteins encoded by the different mutants disappeared at a similar rate. Surprisingly, the wild-type FGF-5 disappeared faster than the mutants. Brefeldin A, which blocks transport of secretory proteins through the Golgi apparatus, moderately reduced the disappearance of the FGF-5 proteins encoded by the different constructs. This excluded a major impact of secretion, and confirmed that intracellular degradation was primarily responsible for the disappearance of the protein. Although the reason for the faster degradation of the wild-type protein is unclear, the similar degradation of the various mutant proteins supports the conclusion that the better production of the spliced peptide from the short mutant proteins is due to the shorter length of the intervening segment favoring the splicing reaction.

Because splicing can occur between fragments that are 40 amino acids apart in the parental protein, we asked whether splicing could also occur between fragments located in distinct parental proteins. Such *trans*-splicing is conceivable in theory, since it was shown that the inner channel of the proteasome, with a diameter of 13 Å (17), can accommodate more than one polypeptide chain (18, 19). The proteasome was also found able to bind and process two substrates simultaneously, one in each catalytic chamber (20). To test this hypothesis we designed plasmids encoding full-length FGF-5 proteins containing a point mutation either in fragment NTYAS or in fragment PRFK. The mutations were chosen so as to abolish the recognition of the corresponding spliced peptide by CTL C2 (8). They were located within the fragments, so as to minimize cleavage site changes. The first mutant (named *FGF-5 Y174A*) coded for alanine instead of tyrosine in fragment NTYAS; the other mutant coded for alanine instead of arginine in fragment PRFK (*FGF-5 R218A*). Upon co-transfection of both mutants, the only way to produce the spliced antigenic peptide would be through splicing of fragments NTYAS and PRFK from two distinct parental proteins. Before testing whether splicing could occur between these two mutated proteins, we verified that the proteasome was able to produce the spliced antigenic peptide in vitro, by incubating the two corresponding mutated precursor peptides with purified 20S proteasomes. The digests obtained were pulsed onto HLA-A3⁺ EBV-

A

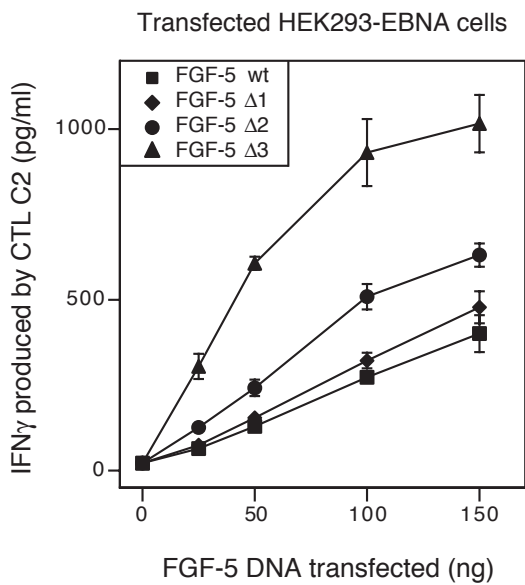
FGF-5 wt : ...¹⁷²**NTYASAIHRTEKTGREWYVALNKR**GKAKRGCSPRVKPQHISTHFL**PRFK**²²⁰...

FGF-5 Δ1 : ...¹⁷²**NTYASAIHRTEKTGREWYVA**_____CSPRVK**PQHISTHFLPRFK**²¹⁰...

FGF-5 Δ2 : ...¹⁷²**NTYASAIHRTEKTGR**_____KPQHISTHFL**PRFK**²⁰⁰...

FGF-5 Δ3 : ...¹⁷²**NTYASAIHR**_____THFL**PRFK**¹⁸⁸...

B



C

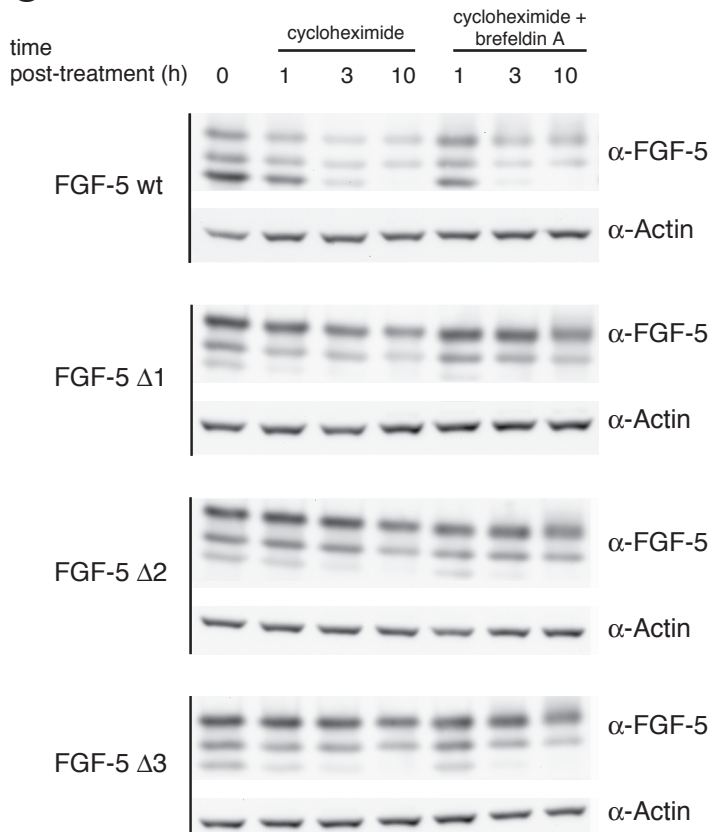


Figure 5. Effect of the length of the intervening sequence on the efficiency of production of the spliced FGF-5 peptide.

(A) Shortened FGF-5 constructs (Δ1 to Δ3) were prepared by PCR from the wild-type FGF-5 (FGF-5 wt) using specific primers for each internal deletion. Each PCR product was cloned into eukaryotic expression vector pEF6.

(B) HEK293-EBNA cells (HLA-A3⁺) were transiently transfected with increasing amounts of each FGF-5 cDNA construct and tested for CTL recognition by CTL C2. Error bars show standard deviations of triplicates.

(C) The disappearance of the different FGF-5 mutant proteins was evaluated by Western blot on cell lysates. HEK293-EBNA cells were transiently transfected with each FGF-5 construct and treated with cycloheximide with or without brefeldin A during the indicated time.

B cells and tested for CTL recognition. As expected, incubations of each mutated precursor alone with 20S proteasomes did not lead to any CTL activation (Fig. 6A). However, when these two mutated precursors were incubated simultaneously with 20S proteasomes, the corresponding digests were able to activate CTL C2. These results indicated that the FGF-5 spliced peptide can be efficiently produced in vitro from these two different mutated precursor peptides. Splicing from distinct peptides also occurs in the cell, as we obtained similar results when we introduced these mutated precursor peptides into COS-A3 cells by electroporation (Fig. 6B). To determine whether splicing can occur in vivo between fragments of two proteins, the mutated plasmids *FGF-5 Y174A* and *FGF-5 R218A* were co-transfected in COS cells, together with a plasmid coding for HLA-A3. As shown on Fig. 6C, transfection of wild-type *FGF-5* led to strong activation of CTL C2 whereas transfection of each mutated construct did not activate the CTL, as expected. When the two mutated *FGF-5* constructs were transfected together, a very weak activation of CTL C2 was detected. Although this activation was significantly higher than the background, it was about 30-fold lower than the one triggered by transfection of wild-type *FGF-5*. We concluded that *trans*-splicing of the *FGF-5* peptide from two distinct protein substrates can occur but with a very low efficiency compared to splicing from a single protein substrate.

Using the same approach, we also tested the efficacy of *trans*-splicing in the case of the spliced gp100 peptide RTKQLYPEW presented by HLA-A32 (9) (Fig. 1). We designed plasmids encoding full length gp100 proteins containing a point mutation either in fragment RTK or in fragment QLYPEW, so that CTL recognition of the corresponding spliced peptides was abolished. The first mutant (*gp100 T41A*) coded for alanine instead of threonine in fragment RTK, and the other mutant coded for alanine instead of glutamate in fragment QLYPEW (*gp100 E51A*). Again, we verified that the proteasome was able to produce the spliced antigenic peptide in vitro, by incubating the corresponding mutated precursor peptides, RAKAWNRQLYPEW and RTKAWNRQLYP Δ W with purified 20S proteasomes. The digests obtained were pulsed onto HLA-A32⁺ EBV-B cells and tested for CTL recognition. As expected, incubations of each mutated precursor alone with 20S proteasomes did not lead to significant CTL activation (Fig. 7A). However, when these two mutated precursors were incubated simultaneously with 20S proteasomes, the corresponding digests were able to activate CTL 14. Thus, the spliced peptide RTKQLYPEW can be efficiently produced in vitro from these two different mutated precursor peptides. Splicing from distinct peptides was also observed in vivo after electroporation in the cell (Fig. 7B). Finally, to determine whether *trans*-splicing can occur in vivo between fragments of two gp100 proteins, mutated plasmids *gp100 T41A* and *gp100 E51A* were co-transfected in

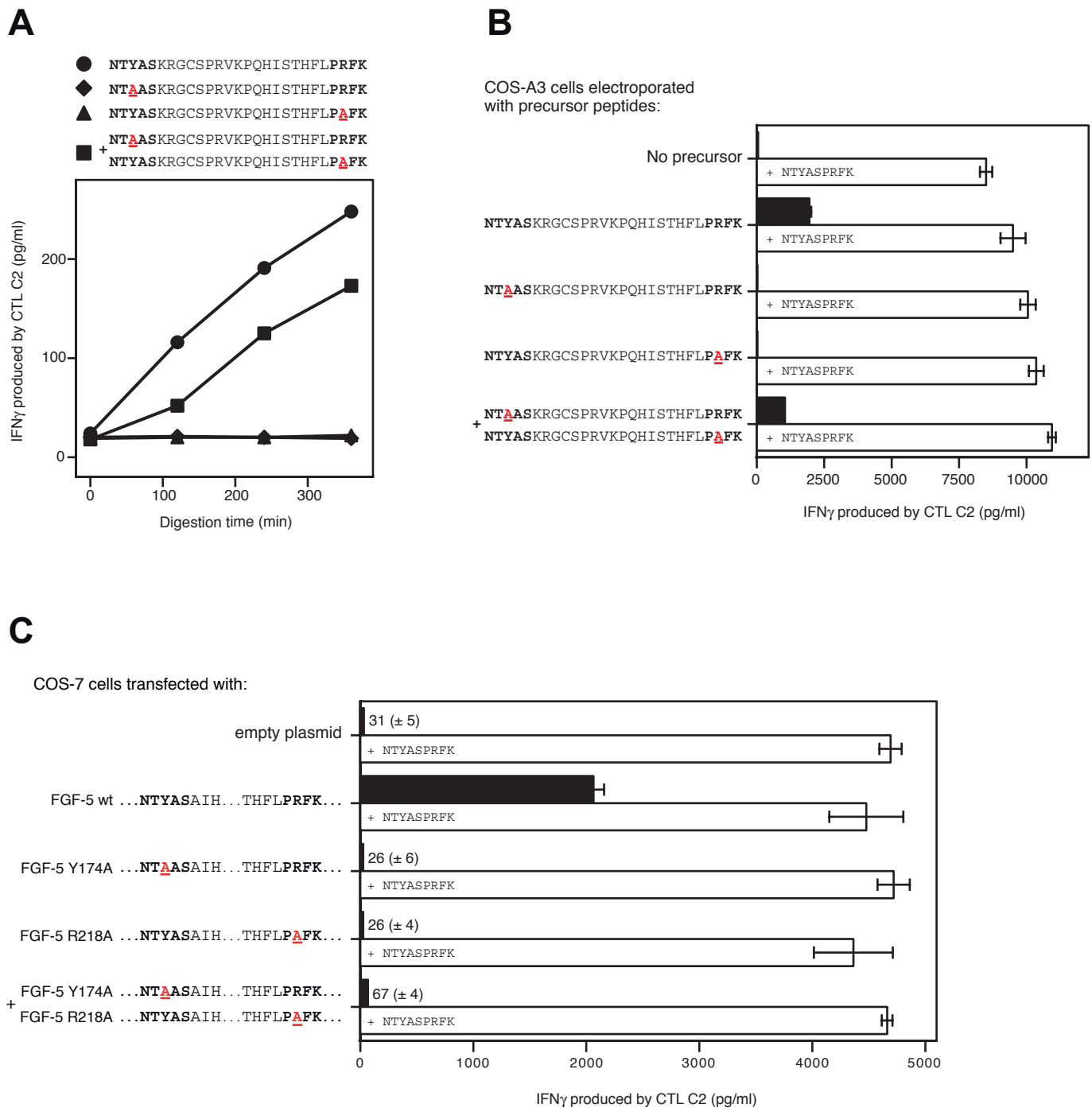


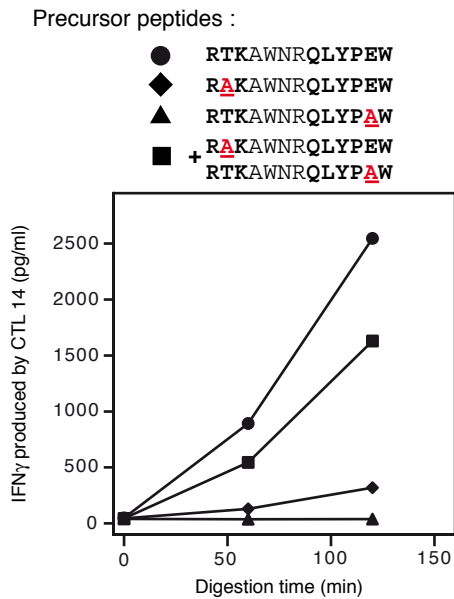
Figure 6. Assay for *trans*-splicing from two distinct peptides or proteins in the case of the spliced FGF-5 peptide.

(A) CTL recognition of digests obtained by *in vitro* incubation of 20S proteasomes with the indicated precursor peptides of the spliced antigenic peptide NTYASPRFK. Reaction was stopped after 120, 240 and 360 min of digestion. The digests were then loaded on HLA-A3⁺ EBV-B cells before addition of CTL C2.

(B) COS-7 cells stably transfected with HLA-A3 were electroporated with the indicated precursor peptides and tested for recognition by CTL C2. Where indicated, cells were also loaded with exogenous peptide NTYASPRFK to control cell viability. Error bars show standard deviations of triplicates.

(C) COS-7 cells were transiently transfected with wild-type or mutated full length FGF-5 cDNA constructs, together with an HLA-A3 cDNA construct. Transfected cells were then tested for recognition by CTL C2. Where indicated, cells were also loaded with exogenous peptide NTYASPRFK. Error bars show standard deviations of triplicates.

COS cells, together with a plasmid coding for HLA-A32. As shown on Fig. 7C (left panel), transfection of wild-type *gp100* led to strong activation of CTL 14. However, no CTL activation was detected after co-transfection of the two mutated *gp100 T41A* and *gp100 E51A*. The mutant constructs were functional, as they encoded another peptide derived from a different region of gp100 equally well as the wild-type *gp100* (Fig. 7C, right panel). Similar results were obtained by transfecting HEK293-EBNA cells or by using another pair of mutant gp100 constructs encoding RTA and QLYPE (data not shown). We concluded that *trans*-splicing of fragments from distinct gp100 proteins does not occur in vivo at a level that is sufficient for recognition of the spliced peptide by our CTL.

A**B**

COS-A32 cells electroporated
with precursor peptides :

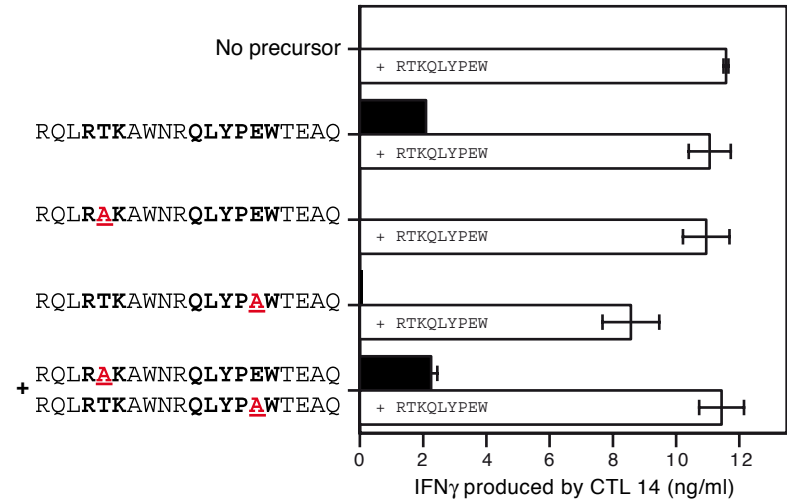
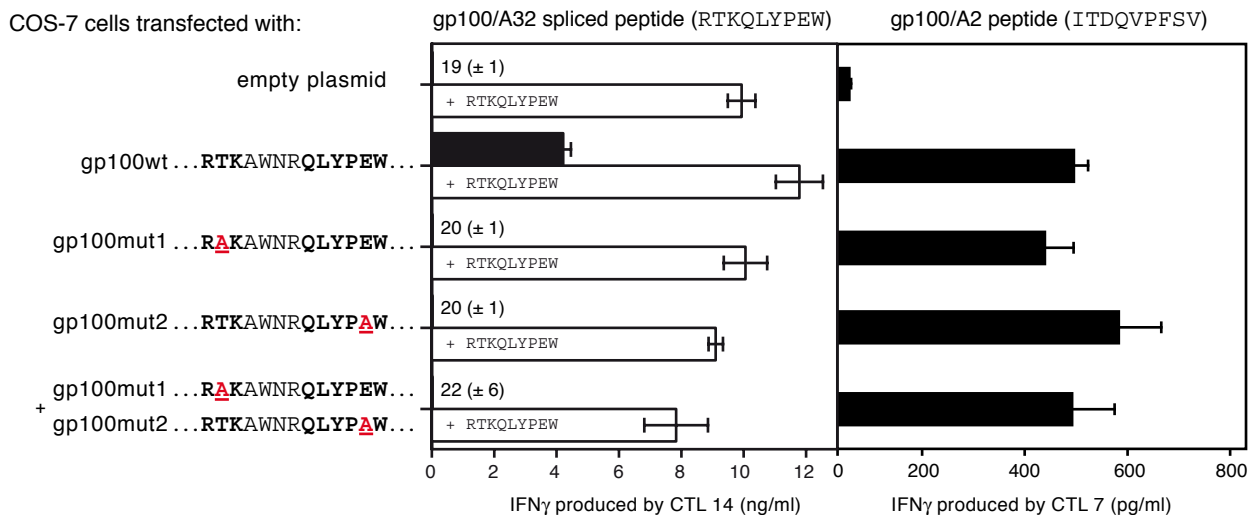
**C**

Figure 7. Assay for *trans*-splicing from two distinct peptides or proteins in the case of the spliced gp100 peptide.

(A) CTL recognition of digests obtained by in vitro incubation of 20S proteasomes with wild-type or mutated precursor peptides of the spliced antigenic peptide gp100_{40-42/47-52} RTKQLYPEW. Reaction was stopped after 60 and 120 min of digestion. The digests were then loaded on HLA-A32⁺ EBV-B cells before addition of CTL clone 14, which recognizes gp100 peptide RTKQLYPEW.

(B) COS-7 cells stably transfected with HLA-A32 were electroporated with the indicated precursor peptides and tested for recognition by CTL 14. Where indicated, cells were also loaded with exogenous peptide RTKQLYPEW to control cell viability. Error bars show standard deviations of triplicates.

(C) COS-7 cells were transiently transfected with wild-type or mutated full length gp100 cDNA constructs, together with an HLA-A32 (*left panel*) or an HLA-A2 cDNA construct (*right panel*). Transfected cells were then tested for recognition by CTL clone 14 (*left panel*; spliced peptide gp100_{40-42/47-52} RTKQLYPEW) or CTL 7, which recognizes peptide gp100₂₀₉₋₂₁₇ ITDQVPFSV presented by HLA-A2 (*right panel*). Where indicated, cells were also loaded with exogenous peptide RTKQLYPEW (*left panel*). Error bars show standard deviations of triplicates.

DISCUSSION

By confirming the direct role of the proteasome in the production of the spliced antigenic peptide derived from FGF-5, our results further strengthen the notion that peptide splicing by the proteasome contributes to the diversity of the peptide repertoire presented by MHC class I molecules. We also confirm the catalytic mechanism we proposed for the two other spliced antigenic peptides, which is a transpeptidation occurring at the active N-terminal threonine of a β -catalytic subunit of the proteasome. During cleavage of the peptide bond, the hydroxyl group of the side chain of this threonine makes an ester bond with the C-terminus of the N-terminal peptide fragment produced by the cleavage, which then remains transiently linked to the proteasome subunit as an acyl-enzyme intermediate. During normal proteolysis, this acyl-enzyme intermediate is rapidly hydrolyzed and the fragment is liberated. In the splicing reaction, the N-terminus of another peptide fragment competes with water molecules and occasionally performs a nucleophilic attack of the acyl-enzyme intermediate, resulting in a transpeptidation reaction that produces the final antigenic peptide. One implication of this mechanism is that it is not determined by a particular sequence motif, but could occur at any major cleavage site by the proteasome, a prediction that we previously confirmed in the model of the gp100 spliced peptide (9).

Another prediction of this catalytic model is that the efficiency of splicing depends on the power with which the free peptide fragment competes with water molecules for the nucleophilic attack of the acyl-enzyme intermediate. Here several factors need to be considered. One is imposed by the structure of the catalytic chamber of the proteasome, which is confined and prevents rapid diffusion of the peptide fragments liberated by the action of the catalytic subunits. The relative concentration of these fragments is thereby significantly increased, favoring transpeptidation over hydrolysis.

A second factor to consider in evaluating the efficiency of production of a given spliced peptide is the probability of interaction of the two relevant fragments. This probability is higher for peptide fragments that are produced simultaneously in the catalytic chamber. It is also dependent on the half-life of the acyl-enzyme intermediate (9, 21). The probability of transpeptidation should then be higher for peptide fragments that are not too distant in the parental protein, as they are more likely to be produced almost simultaneously during degradation of the protein,

which is considered to occur mostly in a processive manner (22). However, we describe here efficient splicing between FGF-5 fragments that are located forty amino acids apart in the parental protein. We observed, as expected, that reducing the length of this intervening segment increased the splicing efficiency. The observed splicing from the wild-type FGF-5 seems to imply that the acyl-enzyme intermediate NTYAS-proteasome is particularly stable, and can survive the time required for production of fragment PRFK. As suggested by Borissenko and Groll, this could result from a high affinity of fragment NTYAS for the non-primed substrate binding channel, which accommodates the peptidic sequence at the N-terminal side of the cleavage site (Fig. 4) (21, 23). But it can also depend on the affinity of fragment AIHRTE for the primed substrate-binding channel, which accommodates the peptidic sequence at the C-terminal side of the cleavage site, as this fragment most likely needs to detach from this channel for fragment PRFK or H₂O to come in and initiate the nucleophilic attack. However, depending on the sense of processive degradation of FGF-5, it is also possible that PRFK is produced first and maintained within the catalytic chamber until the acyl-enzyme intermediate involving NTYAS is formed.

A third factor is the nucleophilicity of the attacking peptide fragment. For FGF-5, the amino terminus of the attacking fragment is the secondary amine of the proline. Compared to the primary amine of other residues, the N-terminus of proline appears to be less reactive for nucleophilic attack, due to a balance between the electron-releasing inducing effects and the steric effects of the cycle (24), and because of a higher pK_a resulting in more protonated forms at physiological pH. Yet the proline N-terminus is still substantially more nucleophilic than water molecules. It was proposed that efficient splicing is dependent on a proper positioning of the attacking fragment in the primed substrate-binding channel (23). Such positioning of the attacking peptide may allow the activation of its amino terminus by a basic amino group present in the catalytic pocket, which may increase its nucleophilicity by attracting a proton, as described for the activation of the water molecule during hydrolysis of the acyl-enzyme intermediate (4). It is possible that fragment PRFK has a particularly high affinity for the primed substrate-binding channel of the proteasome, and that this affinity contributes to explain the splicing efficiency of the FGF-5 peptide despite the distance between the fragments.

We also examined the possibility of peptide splicing between two distinct substrates in living cells. Using synthetic peptides as substrates, which were either digested in vitro with purified proteasomes (Fig. 4B, 6A, 7A and S1A) or electroporated in living cells (Fig. 6B and 7B), we

observed that the proteasome was intrinsically capable of transpeptidation between distinct substrates. In these experiments, the *trans*-splicing observed between distinct peptides was almost as efficient as the splicing from a single substrate. However, using cells expressing pairs of full-length mutated proteins as substrates, we detected a very weak production of the FGF-5 spliced peptide (Fig. 6C) and no production of the gp100 spliced peptide (Fig. 7C). The level of CTL activation obtained with cells transfected with the two FGF-5 mutants together was about thirtyfold lower than with the wild-type FGF-5. These results indicate that *trans*-splicing between distinct proteins is far less efficient than splicing from a single protein substrate, and is unlikely to contribute significantly to the natural production of the FGF-5 spliced antigenic peptide. This might result from the fact that, among the numerous proteins being degraded by the proteasome in the cell, the chance of having precisely the two relevant proteins processed together in the same proteasome particle is very low. Additionally, for reasons of steric hindrance, it might be more difficult for two distinct proteins to fit together within a given proteasome than it is for two peptides. This might explain why *in vivo trans*-splicing between electroporated peptides is more efficient than *in vivo trans*-splicing between full-length proteins. If and when two relevant proteins are processed together, the intrinsically low splicing efficiency seems to result in the production of the relevant spliced peptide at a level that remains below or just above the threshold for CTL recognition. We conclude that most spliced antigenic peptides are expected to comprise fragments of the same parental protein, but these fragments can be distant from each other in the parental protein.

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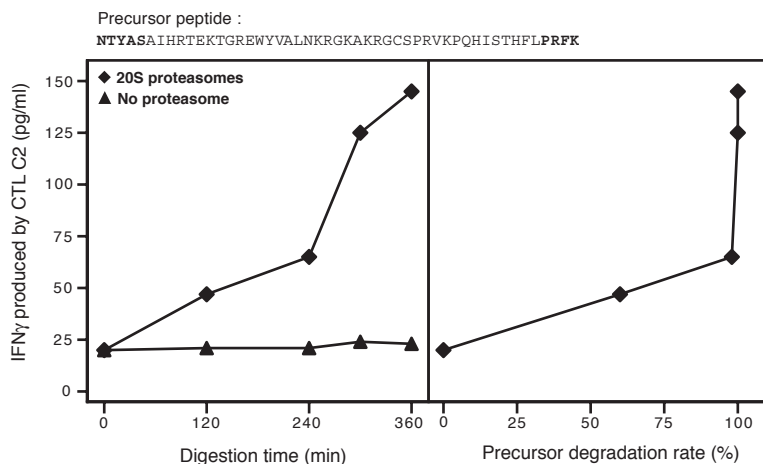
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SUPPLEMENTARY MATERIAL

A



B

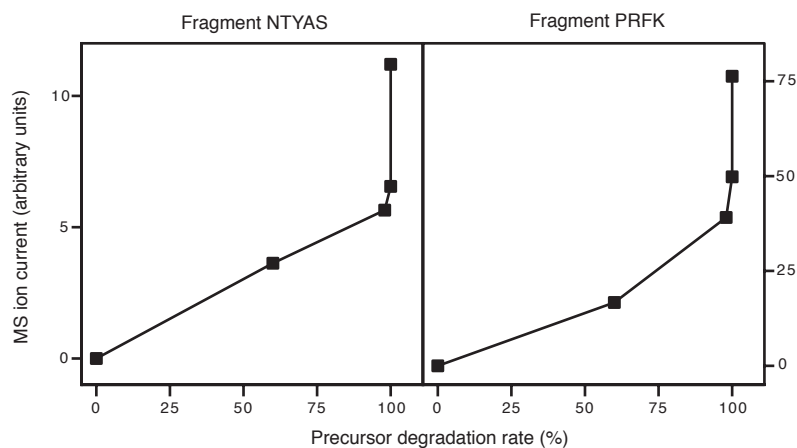


Figure S1. In vitro production of the spliced FGF-5 antigenic peptide by proteasomes.

(A) Twenty micrograms of 49-mer precursor peptide were incubated with 2 μ g of purified 20S standard proteasomes in 40 μ l of Tris 10 mM pH 8 at 37°C. The left panel displays the CTL recognition of digests obtained after the indicated incubation times. The right panel shows the same results in relation to the turnover of the precursor peptide, which was determined by MS.

(B) Production of fragments NTYAS (*left panel*) and PRFK (*right panel*) during the digestion of the 49-mer precursor peptide shown in A. The abundance of each fragment was determined by MS and was shown in relation to precursor peptide turnover determined in A.

II) ARTICLE 2

DIFFERENCES IN THE PRODUCTION OF SPLICED ANTIGENIC PEPTIDES BY THE STANDARD PROTEASOME AND THE IMMUNOPROTEASOME

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Key words: peptide splicing, proteasome, mass spectrometry.

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Abbreviations: IP: immunoproteasome; MS: mass spectrometry; SP: standard proteasome.

Summary

Peptide splicing allows the production of antigenic peptides made of two fragments initially non-contiguous in the parental protein. The proposed mechanism of splicing is a transpeptidation occurring within the proteasome. Three spliced peptides, derived from FGF-5, melanoma protein gp100 and nuclear protein SP110, have been described. Here, we compared the production of these spliced peptides by the standard proteasome and the immunoproteasome. Differential isotope labelling was used to quantify by mass spectrometry the fragments contained in digests obtained with precursor peptides and purified proteasomes. The results show that both standard and immunoproteasomes can produce spliced peptides although they differ in their efficiency of production of each peptide. The FGF-5 and gp100 peptides are more efficiently produced by the standard proteasome, whereas the SP110 peptide is more efficiently produced by the immunoproteasome. This seems to result from differences in the production of the two splicing partners, which depends on a balance between cleavages liberating or destroying those fragments. By showing that splicing depends on the efficiency of production of the splicing partners, these results also support the transpeptidation model of peptide splicing. Furthermore, given the presence of immunoproteasomes in dendritic cells and cells exposed to IFN- γ , the findings may be relevant for vaccine design.

Introduction

Spliced peptides comprise two non-contiguous fragments of the parental protein that are linked together after the excision of an intervening segment. Three spliced antigenic peptides have been described to date. The first is a 9-mer (NTYASPRFK) derived from FGF-5 and presented by HLA-A3 molecules [1]. It was identified using CTL raised against a human renal carcinoma. Another spliced peptide (RTKQLYPEW) is produced from the melanosomal protein gp100 and is recognized, in association with HLA-A32, by CTL raised against a human melanoma [2]. The third example of spliced peptide (SLPRGTSTPK) is a human minor histocompatibility antigen created by a polymorphism in the *SP110* gene [3]. This peptide is recognized, in association with HLA-A3, by CTL isolated from a myeloma patient after MHC-matched hematopoietic cell transplantation.

We previously showed that the three spliced peptides were produced by transpeptidation inside the catalytic chamber of the proteasome [2-4]. In the course of proteolysis, the hydroxyl group of the N-terminal threonine of a catalytic β -subunit produces a nucleophilic attack on the peptide bond, leading to the formation of an acyl-enzyme intermediate between the N-terminal peptide fragment and the proteasome. Normally, the acyl-enzyme intermediate is rapidly hydrolysed by a water molecule present at the active site. However, in the confined catalytic chamber of the proteasome, the acyl-enzyme intermediate is surrounded by a number of peptide fragments, whose N-terminal amino group can compete with water molecules and occasionally produce a nucleophilic attack on the intermediate, thereby creating a new spliced peptide by transpeptidation.

In the present study, we compared the production of the three spliced antigenic peptides by the standard proteasome and its IFN γ -inducible counterpart, the immunoproteasome, using a cellular approach and an in vitro approach. We used differential isotope labelling to perform by mass spectrometry a direct and quantitative comparison of the peptide fragments produced during in vitro digestions.

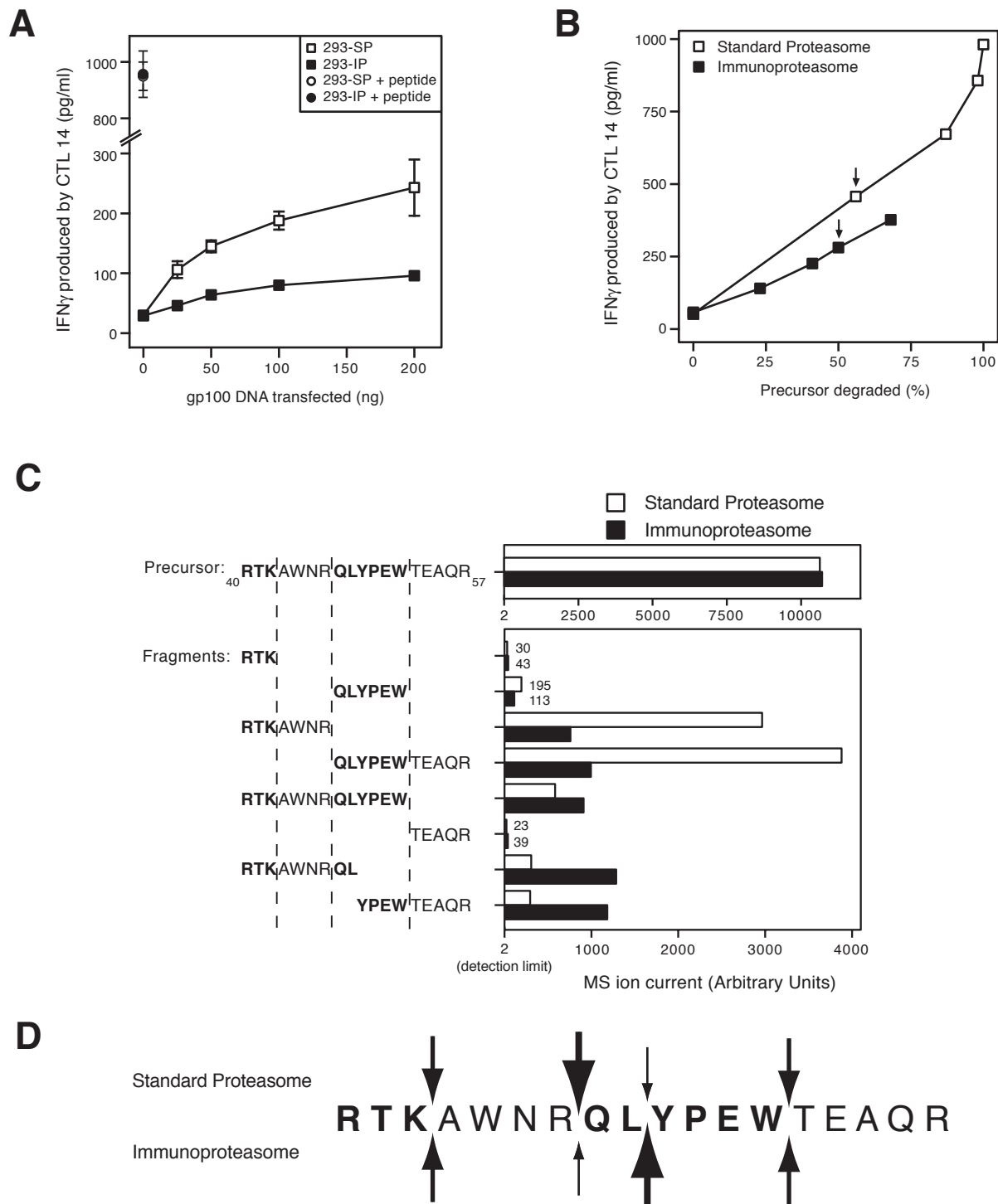


Figure 1. Peptide gp100_{40-42/47-52} RTKQLYPEW is processed more efficiently by the standard proteasome.

(A) Presentation of peptide RTKQLYPEW by cell lines 293-SP and 293-IP. Cells were transfected with 50 ng of a HLA-A32 plasmid construct and the indicated amount of plasmid encoding gp100. The specific CTL clone was added and IFN- γ production was measured 15 hours later in culture supernatants. Control cells were transfected with the HLA-A32 plasmid construct and pulsed or not with the antigenic peptide RTKQLYPEW (2 μ g/ml) for 1 h before addition of the CTL. Error bars show standard deviations of triplicates. One of five similar experiments is shown. (B) CTL recognition of digests obtained by incubation of precursor peptide RTKAWNRQLYPEWTEAQR with purified 20S SP and IP. The reaction was stopped at 0, 20, 40, 60 and 90 minutes of incubation with SP, and 0, 60, 120, 180 and 240 minutes of incubation with IP. Digests were loaded onto HLA-A32+ EBV-B cells and the specific CTL clone was added. IFN- γ production was measured after an overnight coculture and degradation of the precursor peptide was evaluated by MS. One of four similar experiments is shown. (C) Quantitative analysis of the fragments present in the 20-min SP digest and the 180-min IP digest (indicated by an arrow in B). These digests were differentially acetylated with regular or deuterium-labelled acetic anhydride, pooled together and the relative abundance of the precursor peptide and the fragments that are relevant for the production of the antigenic peptide was evaluated by HPLC-MS. (D) Cleavages deduced from C are represented by arrows above the sequence of the precursor for the SP digest and below for the IP digest. The relative efficiency of cleavage by SP and IP at a given site is indicated by the size of the arrow. One of two similar experiments is shown.

Results and discussion

Processing of spliced peptide gp100_{40-42/47-52}

Production of the spliced gp100 peptide RTKQLYPEW involves the excision of four amino acids and the splicing of fragments RTK and QLYPEW by the proteasome [2]. We first studied the presentation of the gp100 spliced peptide in two HEK-293 cell lines engineered to contain exclusively either standard proteasomes (293-SP) or immunoproteasomes (293-IP) [5]. These cells were transiently transfected with plasmid constructs coding for HLA-A32 and gp100. Both cell types were able to present the antigenic peptide but 293-SP cells stimulated approximately 2 times more efficiently CTL 14 than 293-IP cells (Fig. 1A). Both cell lines stimulated the CTL equally well when transfected with HLA-A32 and loaded with the synthetic peptide. To study in details the processing of the gp100 spliced peptide, we digested synthetic precursor peptide **RTKAWNRLYPEWTEAQR** with purified 20S standard proteasomes (SP) or immunoproteasomes (IP). Digests were tested for their ability to sensitize HLA-A32⁺ target cells to CTL recognition. Degradation of the precursor peptide was determined by mass spectrometry so that CTL activation could be monitored in relation to precursor degradation (Fig. 1B). CTL 14 was slightly more reactive toward SP digests than IP digests. We then analysed the digests by HPLC coupled to mass spectrometry (HPLC-MS), and we used a method based on a differential acetylation of the digests to obtain quantitative information on the relative abundance of each fragment in the digests [6]. Two digests, one obtained with SP and one with IP, presenting a similar level of precursor degradation, were differentially acetylated either with regular acetic anhydride or with deuterium-labelled acetic anhydride. Complete acetylation of the peptide fragments was obtained not only at the N terminus but also on the side chain of lysine and tyrosine residues. This differential isotope labelling induces a mass difference between two identical fragments originating from the two different digests. Both modified digests were then pooled together and analysed in a single HPLC-MS run. This approach allows reliable comparative fragment quantitation, because it avoids the problem of ionization efficiency which may vary between distinct MS analyses. Figure 1C shows the abundance of the fragments that are relevant for the production of the spliced gp100 epitope, as measured by this approach in two digests presenting approximately 50% of degradation of the precursor peptide (indicated by an arrow in figure 1B). The equivalent abundance of the precursor peptide in each digest was also verified and is indicated on top of the figure. Even though the detection of fragment **RTK** was somewhat weak, this peptide appeared slightly more produced by the IP than the SP. The other splicing partner, **QLYPEW**, is better detected and appears more abundant in the SP digest. The

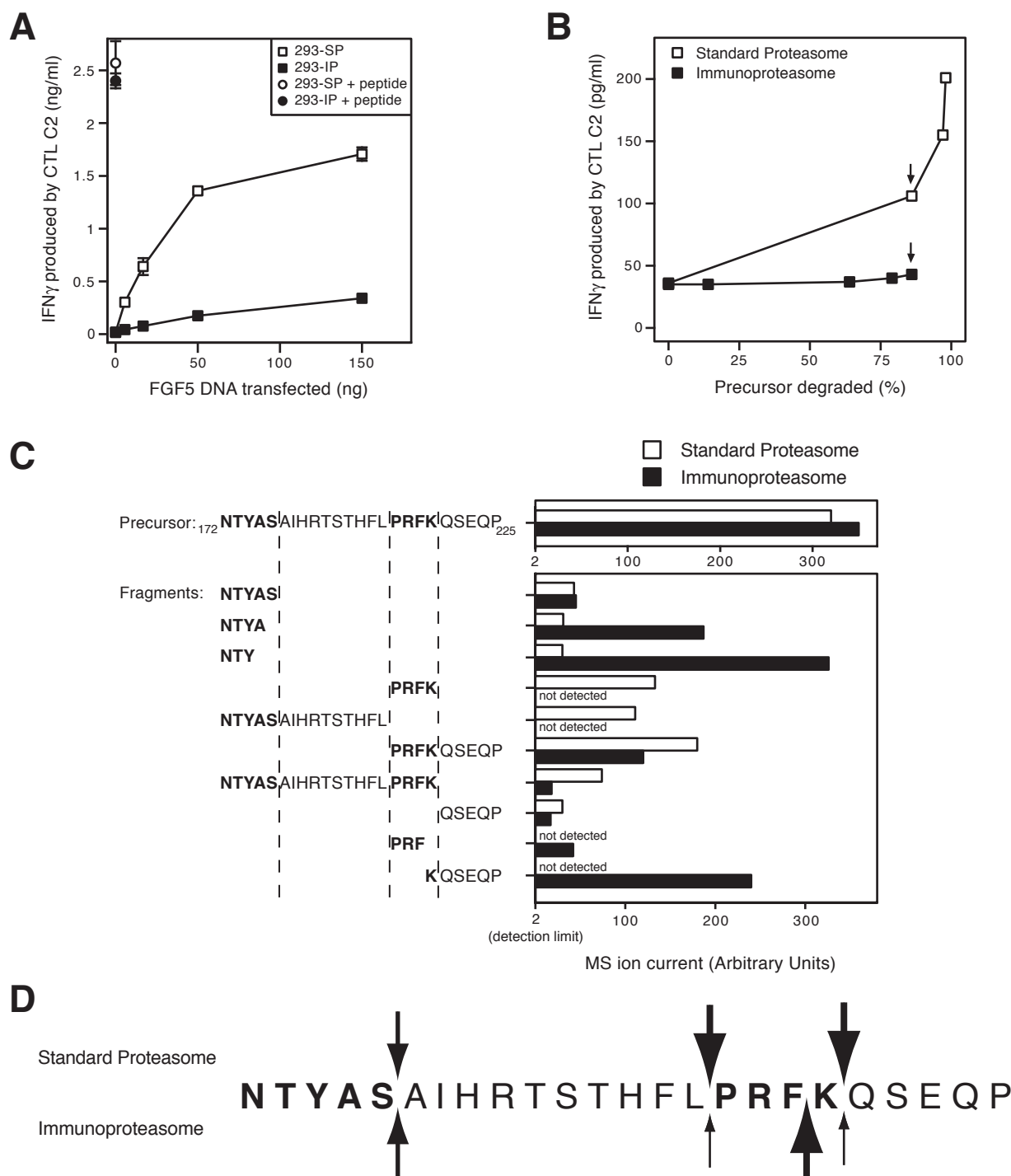


Figure 2. Peptide FGF-5_{172-176/217-220} NTYASPRFK is processed more efficiently by the standard proteasome.

(A) Presentation of peptide NTYASPRFK by cell lines 293-SP and 293-IP. Cells were transfected with the indicated amount of plasmid encoding FGF-5. These cells naturally express HLA-A3. The specific CTL clone was added and IFN- γ production was measured 15 hours later in culture supernatants. Control cells were transfected with an empty plasmid and pulsed or not with the antigenic peptide NTYASPRFK (2 μ g/ml) for 1 h before addition of the CTL. Error bars show standard deviations of triplicates. One of five similar experiments is shown. (B) CTL recognition of digests obtained by incubation of precursor peptide NTYASAIHRTSTHFLPRFKQSEQP with purified 20S SP and IP. The intervening sequence of the FGF-5 precursor was shortened from 40 to 10 amino acids to facilitate MS analysis of the fragments produced. Amino acids were deleted in the middle of the intervening segment to maintain the five residues flanking the two fragments of the antigenic peptide and to minimize potential cleavage modifications at these sites. The reaction was stopped at 0, 120, 240 and 300 minutes of incubation with SP, and also 360 minutes of incubation with IP. Digests were loaded onto HLA-A3+ EBV-B cells and the specific CTL clone was added. IFN- γ production was measured after an overnight coculture and degradation of the precursor peptide was evaluated by MS. One of five similar experiments is shown. (C) Quantitative analysis of the fragments present in the 120-min SP digest and the 360-min IP digest (indicated by an arrow in B). These digests were differentially acetylated with regular or deuterium-labelled acetic anhydride, pooled together and the relative abundance of the precursor peptide and the fragments that are relevant for the production of the antigenic peptide was evaluated by HPLC-MS. (D) Cleavages deduced from C are represented by arrows above the sequence of the precursor for the SP digest and below for the IP digest. The relative efficiency of cleavage by SP and IP at a given site is indicated by the size of the arrow. One of two similar experiments is shown.

efficiency of production of this fragment results from three major cleavages. The cleavage producing the N terminus of fragment **QLYPEW** was about 4 times more important in the SP digest than in the IP digest as shown by the abundance of fragment **RTKAWNR** and its complementary fragment **QLYPEWTEAQR**. Such difference was not observed for the cleavage at the C terminus of **QLYPEW** since fragments **RTKAWNRQLYPEW** and **TEAQR** were slightly more abundant in the IP digest. However, the presence of fragments **RTKAWNRQL** and **YPEWTEAQR** clearly indicated a destructive cleavage within **QLYPEW**, which was about 4 times more important in the IP digest. These differences in efficacy of appropriate or destructive cleavages resulted in a more efficient production of fragment **QLYPEW** in the SP digest.

The comparative abundance of fragments produced by the standard proteasome or the immunoproteasome during these digestions showed that both proteasome types were able to produce the splicing partners RTK and QLYPEW, but fragment QLYPEW was better produced by the standard proteasome. These results are therefore in accordance with those obtained for the CTL assay of the digests, showing a weak difference of production of the gp100 spliced antigenic peptide in favour of the standard proteasome. It should be noted that this MS quantitative analysis did not allow to evaluate the antigenic peptide itself due to its low abundance. This was already the case in our previous studies, in which the presence of spliced peptides could only be detected by the more sensitive tandem MS analysis [2-4].

Processing of spliced peptide FGF-5_{172-176/217-220}

The spliced FGF-5 peptide NTYASPRFK is also produced by transpeptidation in the proteasome despite the long intervening sequence of 40 amino acids separating the splicing partners NTYAS and PRFK [4]. To compare the production of this spliced peptide by the two proteasome types, we first transfected 293-SP and 293-IP cells with a plasmid coding for the entire FGF-5 protein. Transfected 293-SP cells were efficiently recognized by CTL C2 and this recognition was correlated with the amount of FGF-5 transfected (Fig. 2A). However, transfected 293-IP cells were poorly recognized by the CTL. We then incubated synthetic precursor peptide **NTYASAIHRTSTHFLPRFKQSEQP** with purified 20S SP and IP. When loaded onto HLA-A3⁺ target cells, the SP digests were strongly recognized by CTL C2, whereas little or no stimulation of the CTL was observed with the IP digests (Fig. 2B). These results indicated that the FGF-5 peptide was produced almost exclusively by the SP, in accordance with the cellular experiment. Two digests presenting a similar level of precursor degradation were isotope-labelled by acetylation as above, pooled together and analysed by HPLC-MS. Fragment

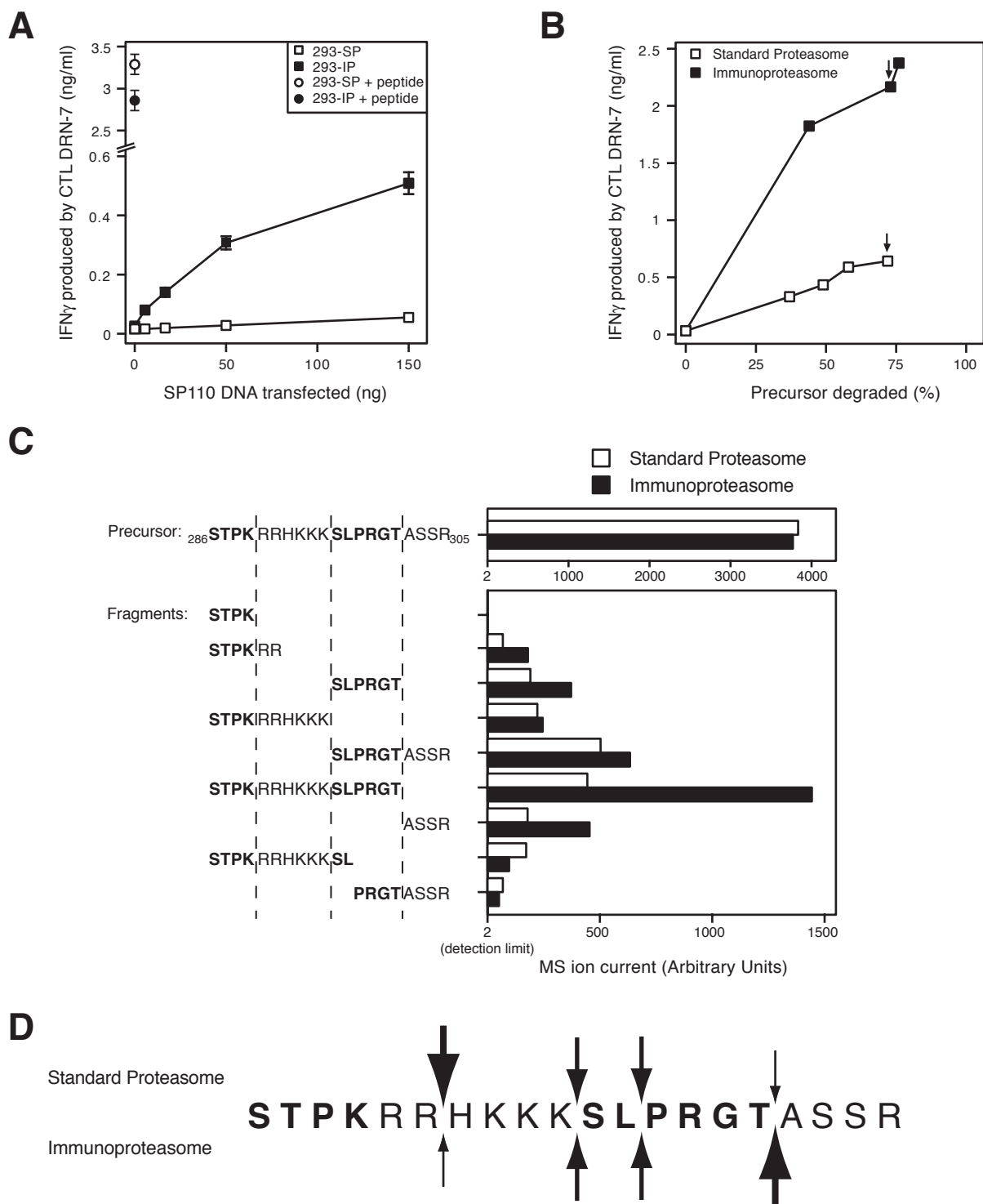


Figure 3. Peptide SP110_{296-301/286-289} SLPRGTSTPK is processed more efficiently by the immunoproteasome.

(A) Presentation of peptide SLPRGTSTPK by cell lines 293-SP and 293-IP. Cells were transfected with the indicated amount of plasmid encoding SP110 protein. These cells naturally express HLA-A3. The specific CTL clone was added and IFN- γ production was measured 15 hours later in culture supernatants. Control cells were transfected with an empty plasmid and pulsed or not with the antigenic peptide SLPRGTSTPK (2 μ g/ml) for 1 h before addition of the CTL. Error bars show standard deviations of triplicates. One of three similar experiments is shown. (B) CTL recognition of digests obtained by incubation of precursor peptide STPKRRHKKKSLPRGTASSR with purified 20S SP and IP. The reaction was stopped at 0, 90, 120, 180 and 240 minutes of incubation for SP digests, and 0, 120, 240 and 300 min of incubation for IP digests. These digests were then loaded onto HLA-A3+ EBV-B cells and the specific CTL clone was added. IFN- γ production was measured after an overnight coculture and degradation of the peptide precursor was evaluated by MS. One of six similar experiments is shown. (C) Quantitative analysis of the fragments present in the 240-min digests (indicated by an arrow in B). These digests were differentially acetylated with regular or deuterium-labelled acetic anhydride, pooled together and the relative abundance of the precursor peptide and the fragments that are relevant for the production of the antigenic peptide was evaluated by HPLC-MS. (D) Cleavages deduced from C are represented by arrows above the sequence of the precursor for the SP digest and below for the IP digest. The relative efficiency of cleavage by SP and IP at a given site is indicated by the size of the arrow. One of three similar experiments is shown.

NTYAS, which composes the first part of the antigen, was equally abundant in the SP and IP digests, despite two stronger destructive cleavages by the IP within this fragment (Fig. 2C). The differential processing of the FGF-5 antigenic peptide was rather explained by the different efficiency to produce the second fragment of the epitope. Indeed, we detected fragment **PRFK** only in the SP digest. The efficiency of production of **PRFK** results from several cleavages. Cleavage at its N-terminus can be monitored with fragment **NTYASAIHRTSTHFL**, which was only detected in the SP digest, and with its complementary fragment **PRFKQSEQP**, which was more abundant in the SP digest. This result indicated that cleavage at the N-terminal side of **PRFK** was better performed by the standard proteasome. The C-terminal cleavage also contributes to a more efficient production of fragment **PRFK** by the SP. Indeed, fragment **NTYASAIHRTSTHFLPRFK** was 4 times more abundant in the SP digest. Furthermore, we observed an internal cleavage within **PRFK**, which was only detected in the IP digest (fragments **PRF** and **KQSEQP**). This cleavage by the IP destroys the second splicing partner of the antigenic peptide.

The different ability of the SP and the IP to perform the N- and C-terminal cleavages of fragment **PRFK**, combined with a destructive cleavage of this fragment by the IP, appear to account for the differential processing of the spliced FGF-5 peptide, which is almost exclusively produced by the SP.

Processing of spliced peptide SP110_{296-301/286-289}

The spliced SP110 peptide **SLPRGTSTPK** comprises two non-contiguous fragments, **STPK** and **SLPRGT**, which are spliced together in the reverse order to that in which they appear in the parental protein [3]. The proteasome was previously shown responsible for the production of this epitope by transpeptidation. We assessed the processing of this spliced peptide by transfecting cell lines 293-SP and 293-IP with the *SP110* cDNA, and testing their CTL recognition. After transfection, 293-IP cells were clearly recognized by CTL DRN-7 whereas 293-SP cells were not (Fig. 3A). We then used purified proteasomes to digest the synthetic 20-amino acid precursor peptide **STPKRRHKKKSLPRGTASSR**. Digests were loaded onto HLA-A3⁺ target cells and tested for CTL recognition (Fig. 3B). IP digests stimulated CTL DRN-7 more efficiently than SP digests, in line with the better processing of the antigenic peptide by 293-IP cells. After differential isotope labelling of two digests presenting a similar level of precursor degradation, we analysed by HPLC-MS the fragments produced. In both digests, we failed to detect fragment **STPK** but we detected fragment **STPKRR** (Fig. 3C). Although the natural peptide eluted from HLA-A3 molecules is **SLPRGTSTPK**, we previously observed that peptide **SLPRGTSTPKRR**

was also recognized by CTL DRN-7 [3]. This C-terminally extended spliced peptide may be produced during in vitro digestion and this was confirmed by HPLC analysis of the digest on a graphitic column: the retention time of the fraction recognized by the CTL was identical to that of synthetic peptide **SLPRGTSTPKRR** (data not shown). This was also the case for another digest obtained with a precursor peptide elongated by four residues at the N-terminal side of **STPKRRHKKKSLPRGTASSR** (data not shown). A potential explanation is that our digestion conditions do not faithfully reproduce what happens in cells where 26S proteasomes process the full-length SP110 protein.

Fragment **STPKRR**, which is the splicing partner in our digests, was more abundant in the IP digest compared to the SP digest (2.5-fold). The other splicing partner, **SLPRGT**, was also more abundant in the IP digest (2-fold). This fragment results from a N-terminal cleavage, which appears to be equal in both proteasome types (fragments **STPKRRHKKK** and **SLPRGTASSR**) and a C-terminal cleavage, which was clearly more efficient in the IP digest as reported by fragments **STPKRRHKKKSLPRGT** and **ASSR**. A destructive cleavage was observed within **SLPRGT** and was found to be somewhat stronger in the SP digest, as indicated by fragments **STPKRRHKKKSL** and **PRGTASSR**.

Altogether, this analysis indicates that the differential processing of the spliced SP110 peptide is mainly explained by a greater capacity of the immunoproteasome to produce the peptide partners of the splicing reaction, at least **SLPRGT**, since we cannot evaluate the “natural” splicing partner **STPK** in our in vitro conditions.

Concluding remarks

Previous studies demonstrated a more efficient production of several antigenic peptides derived from ubiquitous and differentiation self-proteins by the standard proteasome [5, 7, 8]. On the contrary, the immunoproteasome was found more appropriate to produce antigenic peptides derived from pathogens or tumor-specific proteins [9-12]. In the present study, we strengthen these observations with two more examples of antigenic peptides derived from self-proteins that are better produced by the standard proteasome. These are the spliced peptides derived from gp100 and FGF-5. The poor processing of these antigenic peptides by the immunoproteasome could have implications for cancer immunotherapy, as vaccine strategies targeting such antigens should bypass processing in dendritic cells, which mostly contain immunoproteasomes [7, 8, 13, 14]. It may also contribute to explain why CD8⁺ T cells recognizing those peptides, which derive from self proteins, are not deleted in the thymus by negative selection. Ectopic expression of tissue-specific antigens was, indeed, observed in the thymus, which also contains high amounts of immunoproteasomes [15-17].

The comparative analysis of the digests indicates that the production of a given spliced peptide is directly linked to the production of the splicing partner fragments. These findings support the transpeptidation model proposed for the splicing reaction and demonstrate that splicing is not intrinsically favored by specific structural features of a given proteasome type, but is directly dependent on the amount of each peptidic partner produced. Splicing therefore depends on the occurrence of cleavages at appropriate sites to produce the fragments to splice, as predicted by the transpeptidation model.

Materials and methods

Cells lines and CTL clones

Clonal cell lines 293-SP and 293-IP were described previously [5]. CTL clone 14 recognizes the HLA-A32-restricted spliced peptide gp100_{40-42/47-52} RTKQLYPEW [2]. CTL clone C2 recognizes the HLA-A3-restricted spliced peptide FGF-5_{172-176/217-220} NTYASPRFK [1]. CTL clone DRN-7 recognizes the spliced SP110 peptides SLPRGTSTPK and SLPRGTSTPKRR associated with HLA-A3 molecules [3]. These CTLs were expanded as previously described [18] by stimulation with anti-CD3 OKT3 monoclonal antibody (Ortho Biotech Products, Bridgewater, NJ), using irradiated allogeneic PBLs.

Transient transfections and antigen presentation of 293-SP and 293-IP cells

293-SP and 293-IP cells were treated with tetracycline (1 µg/ml) at least 5 days before transient transfections. Cells were transfected using Lipofectamine reagent (Invitrogen) with 50 ng of cDNA corresponding to the appropriate HLA (if not present on HEK-293 cells) and the indicated amounts of cDNAs corresponding to the parental protein. All cDNAs were cloned into plasmid pCDNA3. Twenty-four hours after transfection, the relevant CTL clone was added and after an overnight coculture, the supernatants were collected to measure their IFN-γ content by ELISA (BioSource International, Camarillo, CA).

Proteasome digestions of synthetic peptides and CTL assay

Synthetic peptides were produced using Fmoc solid-phase chemistry. SPs were purified from human erythrocytes and IPs were purified from LCL-721 cells treated with IFN-γ as described before [5]. Digestions of precursor peptides were performed by incubating at 37°C in phosphate buffer (KH₂PO₄/K₂HPO₄ 10 mM pH 7.4), 1 µg of SP or IP with 5 µg of RTKAWNRQLYPEWTEAQR (gp100); 1.5 µg of SP or IP with 7.5 µg of NTYASAIHRTSTHFLPRFKQSEQP (FGF-5); 1 µg of SP or IP with 4 µg of STPKRRHKKKSLPRGTASSR (SP110). Digestions were stopped by addition of trifluoroacetic acid, lyophilized and resuspended in water. One quarter of each digest was diluted in cell culture medium and pulsed in duplicate wells containing 20,000 EBV-B cells for a CTL stimulation assay. We used LG2-EBV-B cells as HLA-A32⁺ target cells and CAN-EBV-B as HLA-A3⁺ cells [3].

Labelling of peptides with acetic anhydride and HPLC-MS analysis

For the gp100 and FGF-5 in vitro experiments, each of the two digests (4 µl) was incubated with 3 µl of NaHCO₃ 1M pH 10.5 and 1 µl of acetic anhydride at room temperature. For the SP110 experiment, 5 µl of each digest were incubated with 8 µl of phosphate buffer 100 mM pH 7.8 and 3 µl of acetic anhydride. The digest obtained with SPs was acetylated with deuterium-labelled acetic anhydride (D₆-Ac₂O) whereas the digest obtained with IPs was acetylated with the regular reagent (H₆-Ac₂O). After 20 minutes, 2 µl of trifluoroacetic acid, or 3 µl in the case of SP110, were added and the two labelled digests were mixed together and subjected to HPLC-MS analysis. The mixed digests were fractionated on a reverse-phase chromatography PepMap C18 0.3/15 (LC Packings, Sunnyvale, CA) with an elution gradient of 5-50% ACN/0.05% TFA (v/v) over 35 min with a flow rate of 4 µl/min. The MS analysis was performed on line with a LCQ Deca XP ion-trap spectrometer equipped with an electrospray ionization source (ThermoFinnigan, San Jose, CA). Relative quantification of the acetylated peptides was done as follows: the differentially labelled ion pairs were first extracted as separate ion chromatograms; corresponding peaks were integrated, and the abundance of each peptide was calculated based on the area under the curve.

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III) ARTICLE 3

AN ANTIGENIC PEPTIDE PRODUCED BY REVERSE SPLICING AND DOUBLE ASPARAGINE DEAMIDATION

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Running title: Spliced and doubly-deamidated antigenic peptide.

Abstract

A variety of unconventional translational and post-translational mechanisms contribute to the production of antigenic peptides, thereby increasing the diversity of the peptide repertoire presented by MHC class I molecules. Here, we describe a class I-restricted peptide that combines several post-translational modifications. It is derived from tyrosinase and is recognized by tumor-infiltrating lymphocytes isolated from a melanoma patient. This unusual antigenic peptide is made of two non-contiguous tyrosinase fragments that are spliced together in the reverse order. In addition, it contains two aspartate residues that replace the asparagines encoded in the tyrosinase sequence. We confirmed that this peptide is naturally presented at the surface of melanoma cells and showed that its processing sequentially requires translation of tyrosinase into the endoplasmic reticulum (ER) and its retrotranslocation into the cytosol, where deglycosylation of the two asparagines by peptide-*N*-glycanase turns them into aspartates by deamidation. This is followed by cleavage and splicing of the appropriate fragments by the standard proteasome, and further transport of the resulting peptide into the ER through the TAP transporter.

Introduction

CD8⁺ cytotoxic T cells (CTLs) are the principal effectors recognizing, through their specific TCR, peptide fragments bound to MHC class I molecules present on the surface of malignant cells. Most of the genes encoding class I-restricted tumor antigenic peptides were discovered by isolating T cells from melanoma patients. After *in vitro* stimulation by autologous melanoma cells, anti-tumor T cells were obtained and used to clone the genes encoding the tumor antigens. These genes included cancer-germline genes, such as those of the MAGE family, which encode tumor-specific antigens expressed in various tumors (Gaugler et al., 1994; Heidecker et al., 2000; Ma et al., 2004; van der Bruggen et al., 1991), but also differentiation genes encoding antigens expressed in both melanomas and normal melanocytes, such as Melan-A (MART-1), gp100 and tyrosinase (Bakker et al., 1994; Brichard et al., 1993; Coulie et al., 1994; Kawakami et al., 1994). Precise identification of the antigenic peptides present at the surface of tumor cells is crucial to develop efficient immunotherapy strategies. These peptides can be used for example to generate melanoma-specific T cells for adoptive immunotherapy or in strategies using epitope-based vaccination. Understanding the processing and presentation pathways of such antigenic peptides may also help designing appropriate protocols of immunotherapy. Class I-restricted antigenic peptides usually consist in fragments of 8-11 amino acids, directly derived from the degradation of the parental protein by the proteasome (Rock et al., 1994). Proteasomal substrates mostly comprise nuclear and cytosolic proteins, but also include endoplasmic reticulum (ER) proteins returned to the cytosol during the process of ER-associated degradation (ERAD) (Bacik et al., 1997; Mosse et al., 1998). In most cases, the sequence of the antigenic peptides can be easily predicted from that of the encoding gene or parental protein. However, a number of antigenic peptides were described whose sequence could not be directly predicted from the encoded sequence. The study of the origin of such non-classical peptides led to the description of unexpected biological mechanisms, acting at a transcriptional, translational or post-translational level. Some peptides were found to arise from aberrant transcription of intronic sequences (Coulie et al., 1995; Guilloux et al., 1996; Robbins et al., 1997) or reverse-strand sequences (Van den Eynde et al., 1999). Others resulted from translation of alternative open-reading frames (Probst-Kepper et al., 2001; Wang et al., 1996), sometimes initiating at non-AUG codons (Malarkannan et al., 1999; Ronsin et al., 1999). Lastly, peptides were found to be modified at the post-translational level, undergoing, for example, deamidation of asparagine residues (Ferris et al., 1999; Selby et al., 1999; Skipper et al., 1996),

phosphorylation of serine and threonine residues (Zarling et al., 2000; Zarling et al., 2006) or peptide splicing (Hanada, Yewdell, and Yang, 2004; Vigneron et al., 2004; Warren et al., 2006). Deamidation was found to occur on asparagine residues corresponding to a *N*-glycosylation site, and is thought to involve retrotranslocation from the ER into the cytosol and deglycosylation by peptide-*N*-glycanase (PNGase) (Altrich-VanLith et al., 2006). Peptide splicing allows the production of antigenic peptides made of two non-contiguous fragments of a given protein that are linked together after the excision of an intervening segment. These two fragments can be linked together either in the order in which they appear in the parental protein, or in the reverse order. Three spliced antigenic peptides were described to date. One is derived from fibroblast growth factor-5 (FGF-5) and is presented by HLA-A3 molecules. It is made of two peptide fragments of five and four residues located forty amino acids apart in the parental protein (Hanada, Yewdell, and Yang, 2004). Another is an HLA-A32-restricted peptide produced from the melanosomal protein gp100 by excision of four amino acids and splicing of the two flanking fragments of three and six residues (Vigneron et al., 2004). The third spliced peptide is a human minor histocompatibility antigen created by a polymorphism in the SP110 gene (Warren et al., 2006). This peptide, presented by HLA-A3 molecules, is made of two non-contiguous fragments of four and six residues, separated by six amino acids in the sequence of SP110 protein. In this case, the two distant fragments are spliced together in the reverse order. The proteasome was found to be responsible for the splicing reaction leading to the production of the three spliced peptides (Dalet et al., 2010; Vigneron et al., 2004; Warren et al., 2006). The catalytic mechanism proposed for the splicing reaction is a transpeptidation occurring at the active N-terminal threonine of a catalytic β -subunit of the proteasome. This transpeptidation involves the nucleophilic attack of the carbonyl carbon atom of an acyl-enzyme intermediate by the N-terminal amino group of a peptide fragment present at the active site of the enzyme. The efficiency of the splicing reaction is very low but is presumably favored in the catalytic chamber of the proteasome due to its confined space. Here, we describe a novel antigenic peptide that contains several post-translational modifications. The peptide, which derives from tyrosinase, is recognized by tumor-infiltrating lymphocytes (TIL) from a melanoma patient. It contains two deamidated asparagine residues and, additionally, is made of two non-contiguous fragments that are spliced in the reverse order. We analyzed in detail the processing pathway leading to the production of this highly unusual peptide.

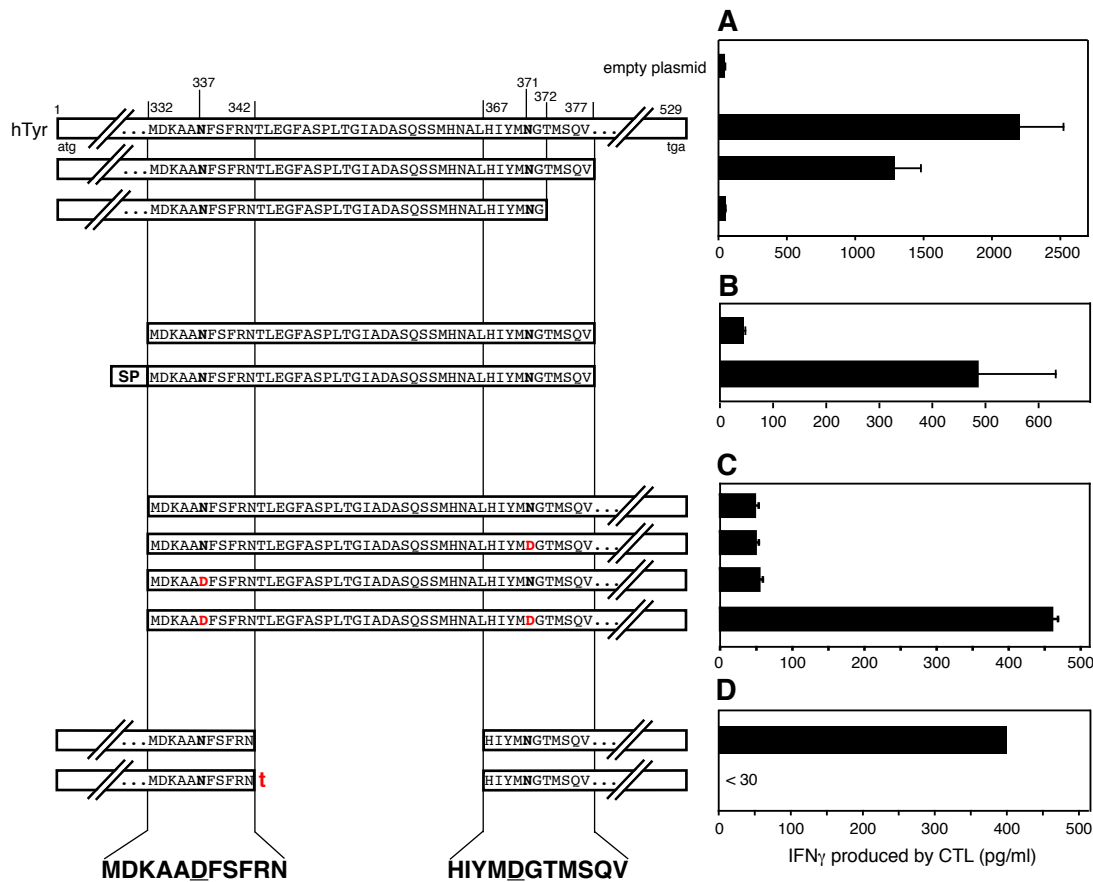


Figure 1. Localization of the minimal region of tyrosinase required to produce the antigen.

(A) (B) (C) COS-7 cells were transiently transfected with a construct encoding HLA-A24 together with the indicated constructs derived from human tyrosinase (hTyr). The day after, these cells were tested for recognition by CTL CL62, a tyrosinase-reactive CTL clone isolated from line TIL888. IFN γ production was measured by ELISA in the culture supernatants after overnight incubation. In (B), SP indicates that the plasmid construct encodes the signal peptide of IL2. In (C), where indicated, the Asn residues at position 337 or 371 were replaced by Asp residues by site-directed mutagenesis.

(D) HEK293-A24 cells were transiently transfected with the indicated tyrosinase constructs containing an internal deletion. Transfected cells were tested for their recognition by TIL888 one day later. Where indicated, an additional thymidine (t) was inserted between the codons corresponding to amino acids 342 and 367. The addition of this nucleotide results in a downstream frameshift after translation of the construct. The sequence of the minimal regions of tyrosinase required for recognition by the CTL is indicated below the sequences of the different constructs tested. Data shown are representative of at least two independent experiments. Error bars show standard deviations of triplicates.

Results

Evidence for ER processing of an HLA-A24 restricted tyrosinase epitope

The adoptive transfer of tumor-infiltrating lymphocyte line TIL888 into the autologous melanoma patient was followed by tumor regression (Robbins et al., 1994). TIL888 was found to recognize a tyrosinase-derived antigen presented by HLA-A24 molecules (Robbins et al., 1994). To localize the peptide recognized by TIL888 and its representative clone CL62, we generated truncated constructs of the human tyrosinase gene and transfected them into COS-7 cells together with a plasmid encoding the HLA-A24 molecule. These cells were then tested for their ability to stimulate CTL CL62. Cells transfected with a construct encoding tyrosinase amino acids 1 to 377 were recognized by CTL CL62 while cells transfected with a shorter construct encoding amino acids 1 to 372 were not (Fig. 1A), in accordance with previous experiments (Kang et al., 1995). The tyrosinase gene encodes a signal peptide targeting translation into the ER where signal peptidase removes the N-terminal signal peptide. To evaluate the importance of this ER transit for production of the antigenic peptide, we generated two shorter constructs encoding amino acids 332 to 377, either alone or preceded by a signal peptide (Fig. 1B). Although COS-7 cells transfected with the construct containing the signal peptide were recognized by CTL CL62, those transfected with the plain construct, which is translated in the cytosol, were not, indicating that translation into the ER was required for production of the antigen.

Involvement of asparagine deamidation in the production of the tyrosinase epitope

The previously described HLA-A*0201 restricted peptide ₃₆₉YMDGTMSQV₃₇₇ contains an Asp residue at position 371 instead of the Asn residue predicted by the nucleotide sequence of the gene (Skipper et al., 1996). The Asn₃₇₁ residue, which represents an *N*-linked glycosylation site, appears to be deglycosylated by the cytosolic enzyme peptide-*N*-glycanase (Altrich-VanLith et al., 2006), a process that occurs in the cytosol after retrotranslocation of tyrosinase from the ER (Mosse et al., 1998). A construct lacking the signal sequence and containing a substitution of Asn₃₇₁ with an aspartic acid was not recognized by CTL CL62 (Fig. 1C). However, this fragment contains another Asn residue at position 337 which contains an *N*-linked glycosylation consensus sequence. A tyrosinase₃₃₂₋₅₂₉ construct encoding the N337D substitution also failed to activate CTL whereas a construct encoding an Asp residue at both positions 337 and 371 was recognized by CTL CL62, indicating that both regions contributed to the epitope recognized by these T cells (Fig. 1C) and providing

evidence that this epitope was processed in a similar way to the previously described HLA-A*0201 restricted ₃₆₉YMDGTMSQV₃₇₇ epitope (Mosse et al., 1998; Skipper et al., 1996). Evaluation of tyrosinase variants containing internal deletions of sequences between Asn337 and Asn371 revealed that transfection of a construct lacking the region that encoded amino acids 343 to 366 stimulated cytokine release from TIL888 (Fig. 1D). A variant containing the same deletion but with an additional thymidine after codon 342, resulting in a downstream frameshift, was not able to sensitize cells to recognition by TIL888 (Fig. 1D). This result provided evidence that RNA splicing was not responsible for linking these two regions, as this process presumably would be unaffected by the addition of a single nucleotide at this position, and raised the possibility that this antigenic peptide was produced by peptide splicing, as described recently (Hanada, Yewdell, and Yang, 2004; Vigneron et al., 2004; Warren et al., 2006).

Evidence for peptide splicing in generating the tyrosinase epitope

Target cells loaded with peptides of varying lengths containing combinations of amino acids between residues 332 and 342 followed by amino acids between residues 367 and 377, and containing the two aspartic residues D337 and D371 failed to be recognized by CTL CL62, even when high concentrations of peptides were used (unpublished data). These peptides only partially fit the HLA-A24 binding motif, namely Y or F at position 2 and F, I or L at the C terminus (Kubo et al., 1994; Maier et al., 1994). However, we observed that many peptides made of two fragments assembled in the reverse order contain more appropriate HLA-A24 binding motifs. We then synthesized and tested the recognition of 180 peptides containing residues from the two tyrosinase regions determined above that were spliced in reverse order and in addition included the two aspartate substitutions at residues 337 and 371 (Fig. S1). To facilitate the synthesis and the subsequent analysis of these peptides, they were prepared in 30 pools of 6 peptides. Each pool was evaluated for its ability to stimulate the CTL. The peptides contained in each of the four positive pools were further fractionated by HPLC and the fractions were tested for CTL recognition. Six fractions, corresponding to six different peptides, were able to stimulate CTL CL62 (Fig. S1). These six peptides were then synthesized separately, purified and tested for CTL recognition after titration. Two of them, with sequences IYMDGTADFSF and IYMDGAADFSF, were efficiently recognized with a half-maximal activation at a peptide concentration comprised between 0.1 and 1 ng/ml (Fig. 2A). The other purified peptides were able to stimulate the CTL but only at higher concentrations.

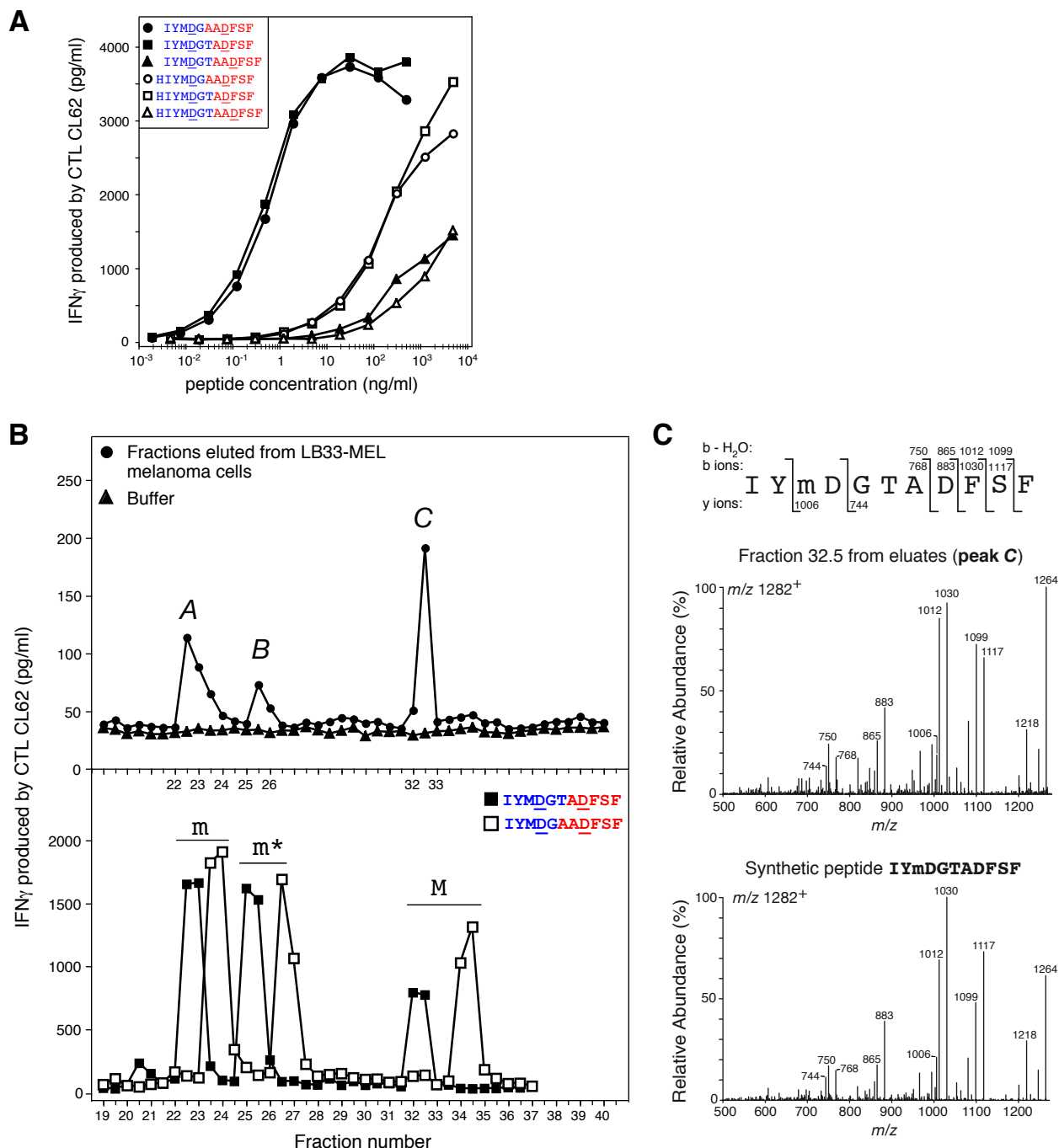


Figure 2. Identification of the antigenic peptide presented by melanoma cells and recognized by CTL CL62.

(A) CTL recognition of synthetic peptides composed of two non-contiguous fragments of tyrosinase that are joined together in the reverse order to that of the tyrosinase sequence. The indicated peptides were loaded onto HLA-A24+ EBV-B cells before the CTL assay. IFN γ production was measured after an overnight incubation.

(B) Peptides were eluted from HLA class I molecules purified from melanoma cells LB33-MEL and were fractionated on a reversed-phase column by HPLC. The fractions were loaded onto presenting cells and tested for CTL recognition (top panel). IFN γ production was measured after an overnight incubation. Three peaks of CTL activity, indicated by A, B and C on the graph, were detected. Buffer was run on the column before the eluted peptides to rule out any contamination of the HPLC system, and the fractions were tested similarly. Synthetic peptides IYMDGTADFSF and IYMDGAADFSF were oxidized by treatment with H₂O₂. The non-oxidized (M), the methionine sulfoxide (m) and the methionine sulfone (m*) forms of the peptides were separated and purified. Each peptide (10 ng of each form) was injected under the same HPLC conditions as those used for eluates, and the fractions were tested for CTL recognition (bottom panel).

(C) MS/MS fragmentation spectrum of the singly charged ion with m/z 1282 detected in fraction 32.5 (peak C) from LB33-MEL eluates (top), and fragmentation spectrum of the singly charged ion (m/z 1282) of the synthetic peptide IYmDGTADFSF (bottom). The fragments that were detected are indicated above the peptide sequence for N-terminal b ions and their dehydrated forms, and below the sequence for C-terminal y ions. Ion with m/z 1264+ is the dehydrated form of ion 1282+ and ion with m/z 1218+ corresponds to the loss of methanesulfenic acid from the methionine sulfoxide of ion 1282+ (Lagerwerf et al., 1996). Data shown are representative of at least two independent experiments.

Identification of the naturally processed tyrosinase peptide

Peptides IYMDGTADFSF and IYMDGAADFSF, which correspond, respectively, to the reverse splicing of fragments ADFSF and IYMDGT or AADFSF and IYMDG, seemed therefore to be the best candidates for the natural antigenic peptide. To determine which candidate was identical to the natural peptide presented by melanoma cells, we isolated MHC class I molecules from melanoma cells LB33-MEL, which were efficiently recognized by CTL CL62. We eluted the peptides bound to MHC molecules with acid, fractionated them on a reversed-phase column by HPLC and tested the collected fractions for CTL recognition. Surprisingly, the fractions recognized by CTL CL62 were distributed in three distinct peaks, designated peak *A*, peak *B* and peak *C* (Fig. 2B, *top panel*). In parallel, we observed by HPLC-MS that the synthetic peptides IYMDGTADFSF and IYMDGAADFSF spontaneously oxidized partially on their methionine residue, whose sulfur atom acquired an oxygen and became a sulfoxide. Under strong oxidative conditions, we observed further oxidation of the methionine sulfoxide into methionine sulfone, with two oxygen atoms on the same sulfur atom. The three forms of each of the two candidates, i.e. non-oxidized, sulfoxide and sulfone, were prepared, purified and chromatographed together under the HPLC conditions used for the peptides eluted from LB33-MEL cells. The collected fractions were then loaded onto target cells and tested for CTL recognition. We observed three peaks of CTL activity for each candidate. By comparing their retention times with those of the purified peptides, we assigned the first peak to the sulfoxide form (m), the second to the sulfone (m*) and the third to the non-oxidized form (M) (Fig. 2B, *bottom panel*). This indicated that the oxidized forms of the candidates were also recognized by CTL CL62. More importantly, the three peaks of CTL activity detected for synthetic peptide IYMDGTADFSF after fractionation corresponded exactly to the peaks obtained with the peptides eluted from melanoma cells (Fig. 2B). This was not the case for the positive peaks obtained after fractionation of the three oxidized forms of synthetic peptide IYMDGAADFSF. Thus, the profile of retention times of the antigenic peptide present at the surface of melanoma cells was identical to that of synthetic peptide IYMDGTADFSF. Notably, a titration of the three purified forms of synthetic peptide IYMDGTADFSF showed that they were equally recognized by the CTL (Fig. S2). We also synthesized and tested spliced peptide ADFSFIYMDGT, made of the same non-contiguous fragments but assembled in the normal order, and peptide IYMNGTANFSF, which contains the two Asn residues of the original sequence of tyrosinase. However, these two peptides were not recognized by the CTL (Fig. S3). Finally, peptide IYMDGTADFSF was clearly

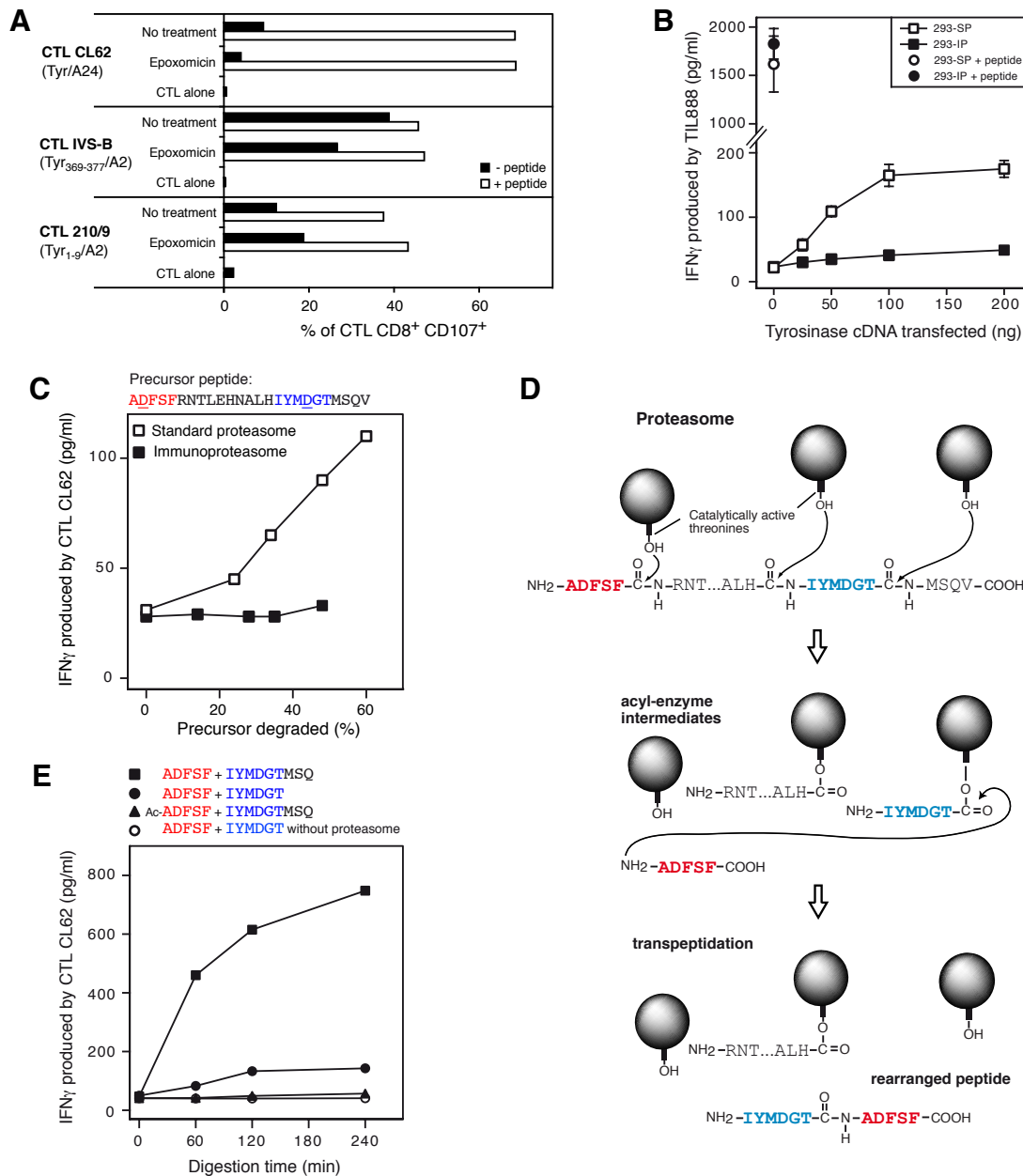


Figure 3. Production of the spliced antigenic peptide IYMDGTADFSF by the proteasome.

(A) CTL recognition of melanoma cells treated or not with proteasome inhibitor. Melanoma cells LB39-MEL naturally expressed tyrosinase, HLA-A24 and HLA-A2. They were treated during 2 hours with epoxomicin (0.5 μ M) after acid elution of MHC-bound peptides. Cells were then washed and incubated during one more hour with a lower dose of epoxomicin (0.01 μ M) and, where indicated (white bars), with the corresponding synthetic antigenic peptide as a control. Recognition by three CTL clones was evaluated by flow cytometry in a degranulation assay performed after 5 hours of incubation with LB39-MEL cells. The percentage of CD8⁺ T cells expressing the degranulation markers CD107a and CD107b is shown. CTL CL62 recognizes tyrosinase/A24 spliced peptide IYMDGTADFSF, CTL IVS-B recognizes tyrosinase/A2₃₆₉YMDGTMSQV₃₇₇ and CTL 210/9 recognizes tyrosinase/A2₁MLLAVLYCL₉. One of three similar experiments is shown.

(B) Processing of peptide IYMDGTADFSF by cells expressing standard proteasomes (293-SP) or immunoproteasomes (293-IP). Both cell lines were transfected with an HLA-A24 plasmid construct and the indicated amounts of plasmid encoding tyrosinase. CTLs TIL888 were added and IFN γ production was measured in supernatants after an overnight incubation. Control cells were transfected with a plasmid coding for HLA-A24 and pulsed with the synthetic peptide IYMDGTADFSF during 1 h before addition of the CTLs. One of two similar experiments is shown. Error bars show standard deviations of triplicates.

(C) In vitro production of the tyrosinase spliced peptide by the standard proteasome. The indicated 25-mer precursor peptide was incubated with purified 20S standard proteasomes or immunoproteasomes. Reaction was stopped after 0, 1, 2, 3 and 4 hours of incubation. Digests were then loaded onto HLA-A24+ EBV-B cells. CTL CL62 was added and IFN γ production was measured after an overnight incubation. The degradation of the precursor peptide was determined by MS. One of two similar experiments is shown.

(D) Model for the catalytic mechanism producing the tyrosinase spliced peptide in the proteasome. The balls represent catalytically active β -subunits of the proteasome with the hydroxyl group of the side chain of their N-terminal threonine.

(E) CTL recognition of digests obtained by incubating purified 20S standard proteasomes with various synthetic peptides during the indicated periods of time. The different digests were loaded onto presenting cells and tested for CTL stimulation. One of four similar experiments is shown. Ac-ADFSF, N-terminally acetylated peptide with sequence ADFSF.

identified in peak *C* of the eluates by using HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) (Fig. 2C). Although the retention time of peak *C* clearly indicated the presence of the non-oxidized form of IYMDGTADFSF in the melanoma eluates, the HPLC-MS/MS analysis of peak *C* only detected this peptide under its sulfoxide form, with m/z 1282 and a fragmentation pattern identical to that of the corresponding synthetic sulfoxide peptide (Fig. 2C). The lack of detection of the non-oxidized form of IYMDGTADFSF in this fraction is likely due to its oxidation after the HPLC separation, probably during the drying step. We also detected by MS/MS the sulfoxide form of the peptide in peak *A* of LB33-MEL eluates, as well as the sulfone form in peak *B*, although the latter was less abundant and close to the detection limit of our system (Fig. S4). Taken together, these results confirmed that the natural antigenic peptide presented by HLA-A24 to CTL CL62 is the 11-mer IYMDGTADFSF, which is a spliced and reordered peptide composed by the two non-contiguous fragments ADFSF and IYMDGT, each of them containing an Asp residue resulting from deamidation of an Asn residue.

Production of the spliced and reordered peptide by the proteasome

The proteasome is responsible for the splicing reaction leading to the production of the three spliced antigenic peptides described so far (Dalet et al., 2010; Vigneron et al., 2004; Warren et al., 2006). We evaluated its involvement in the production of the tyrosinase spliced peptide IYMDGTADFSF by treating melanoma cells LB39-MEL with the specific proteasome inhibitor epoxomicin. These cells naturally express tyrosinase and the two HLA alleles A2 and A24. Recognition of treated cells was evaluated in a degranulation assay, in which CTL activation was determined by measuring the surface expression of degranulation markers CD107a (LAMP1) and CD107b (LAMP2). Treatment with epoxomicin decreased the recognition of melanoma cells by CTL CL62 (Fig. 3A, *upper part*). As control, we also evaluated in the same experiment the effect of epoxomicin on the presentation of two HLA-A2-restricted tyrosinase-derived antigenic peptides, one (₃₆₉YMDGTMSQV₃₇₇) being proteasome- and TAP-dependent, the other (₁MLLAVLYCL₉) being derived from the signal peptide and described as proteasome- and TAP-independent (Wölfel et al., 2000; Wölfel et al., 1994). As expected, epoxomicin treatment decreased the presentation of the former but not the latter (Fig. 3A). Next, we showed that an HLA-A24⁺ tumor cell line, which does not express tyrosinase, was able to sensitize CTL CL62 when electroporated with the 25-residue precursor peptide ADFSFRNTLEHNALHIYMDGTMSQV (Fig. S5). The CTL activation was impaired when these cells were treated with epoxomicin before electroporation of the

precursor. Collectively, these experiments strongly suggested an implication of the proteasome in the production of the spliced peptide IYMDGTADFSF.

Upon exposure of cells to IFN γ , the three catalytic β -subunits $\beta 1$, $\beta 2$ and $\beta 5$ of the constitutively expressed proteasome, namely the standard proteasome, are replaced by their three homologous inducible subunits $\beta 1i$, $\beta 2i$ and $\beta 5i$, to create a slightly different protein complex called the immunoproteasome. The two proteasome subtypes display partially overlapping but different cleavage specificities (Toes et al., 2001), which can result in a different capacity to produce certain antigenic peptide (Chapiro et al., 2006; Dalet et al., 2011; Morel et al., 2000; Van den Eynde and Morel, 2001). The presentation of the tyrosinase spliced peptide by these two proteasome types was evaluated using HEK293 cells engineered to contain exclusively standard proteasomes (293-SP) or immunoproteasomes (293-IP). After transient transfection of these two cell lines with plasmids encoding tyrosinase and HLA-A24, we observed that cells expressing standard proteasomes were efficiently recognized by CTLs, whereas cells expressing immunoproteasomes were not, despite a similar capacity to present the synthetic antigenic peptide when exogenously pulsed on cells (Fig. 3B). These results supported the implication of the proteasome in the production of the tyrosinase spliced peptide, which appeared to be more efficiently produced by the standard proteasome.

To directly evaluate the ability of the proteasome to produce this spliced peptide, we incubated purified 20S proteasomes with the precursor peptide used in the electroporation experiment. The resulting digests were loaded onto presenting cells before testing their ability to stimulate CTL CL62. Digestion of the precursor peptide by 20S standard proteasomes led to a CTL activation increasing with the degradation of the precursor peptide (Fig. 3C). In contrast, the digests obtained with the immunoproteasome failed to activate the CTL. Efficient degradation of the precursor peptide by both proteasome types was verified by MS. These results confirmed that the spliced peptide IYMDGTADFSF is indeed produced by the standard proteasome but not by the immunoproteasome. According to the catalytic mechanism proposed for the previously described spliced peptides, cleavage at the C terminus of fragment IYMDGT would be required to form an acyl-enzyme intermediate, which would be further attacked by the N-terminal amino group of fragment ADFSF to form, by transpeptidation, the rearranged spliced peptide (Fig. 3D). To validate this mechanism, we incubated various pairs of synthetic peptides together with purified 20S standard proteasomes. Digests obtained with peptides ADFSF and IYMDGTMSQ were efficiently recognized by CTL CL62 (Fig. 3E) and the presence of the spliced peptide IYMDGTADFSF was confirmed by MS/MS in the 240-min digest (unpublished data). On the contrary, when we incubated

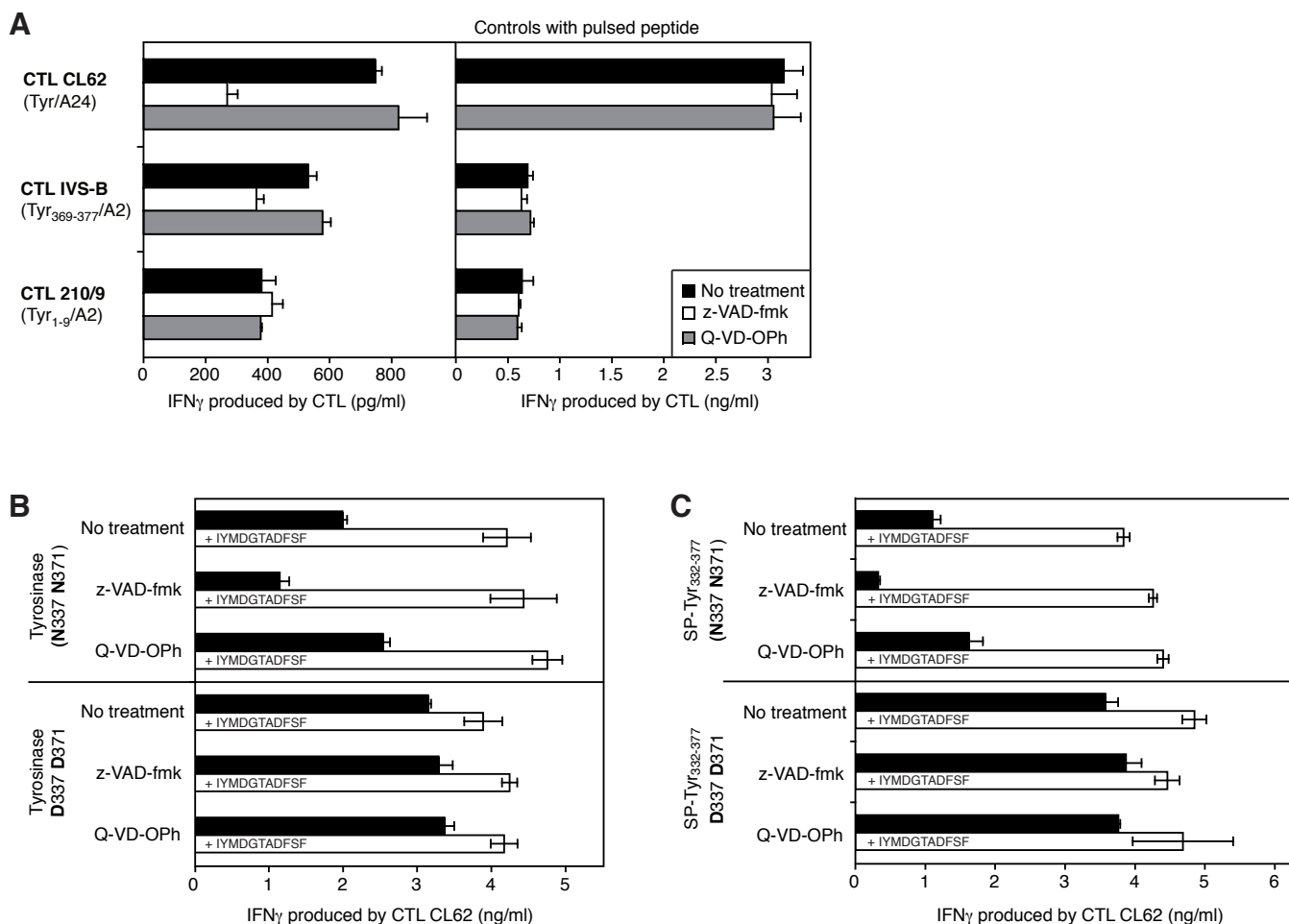


Figure 4. Role of peptide-N-glycanase in the processing of the tyrosinase spliced peptide.

(A) Melanoma cells LB39-MEL, expressing both HLA-A24 and HLA-A2, were treated during 5 hours with the indicated compounds (50 μ M) after acid elution of MHC-bound peptides. Both z-VAD-fmk and Q-VD-OPh are caspase inhibitors. Only z-VAD-fmk blocks PNGase activity. Cells were then tested for recognition by three different CTL clones (left panel). CTL CL62 recognizes the tyrosinase/A24 spliced peptide IYMDGTADFSF, CTL IVS-B recognizes tyrosinase/A2₃₆₉YMDGTMSQV₃₇₇ and CTL 210/9 recognizes tyrosinase/A2₁MLLAVLYCL₉. IFN γ production was measured after an overnight incubation. Cells were pulsed with the corresponding synthetic antigenic peptide before addition of the CTL to check their antigen-presenting capacity (right panel). Data shown are representative of at least two independent experiments.

(B) COS-7 cells were transiently transfected with plasmids encoding HLA-A24 and a full-length tyrosinase construct encoding either Asn residues at positions 337 and 371 (N337 N371), or Asp residues at the same positions (D337 D371). Five hours after transfection, cells were incubated overnight with 50 μ M of the indicated compounds. Supernatants were then removed and CTL CL62 was added. Where indicated (white bars), cells were pulsed during one hour with the antigenic peptide before addition of the CTL.

(C) Same experiment as B except that cells were transfected with constructs coding for tyrosinase₃₃₂₋₃₇₇ preceded by the signal peptide of IL2 (SP-Tyr₃₃₂₋₃₇₇). For B and C, data shown are representative of four independent experiments. In all panels, error bars show standard deviations of triplicates.

proteasomes with peptides ADFSF and IYMDGT, the digests were weakly recognized by the CTL. These results indicated that cleavage of the peptide bond after the Thr of fragment IYMDGTMSQ is required to obtain an efficient production of the spliced peptide IYMDGTADFSF, as predicted by the transpeptidation model. We also observed that production of the spliced peptide was abolished by blocking the N-terminal amino group of fragment ADFSF with an acetyl group. This confirmed that fragment ADFSF is responsible, with its N-terminal amino group, for the nucleophilic attack of the acyl-enzyme intermediate. Collectively, these results confirmed that the proteasome is responsible for the transpeptidation reaction leading to the spliced and reordered peptide IYMDGTADFSF.

Deamidation of asparagine residues by peptide-N-glycanase

Next, we verified whether this spliced antigenic peptide, which contains two Asp residues instead of the two Asn residues present in the tyrosinase sequence, followed the same processing pathway as the tyrosinase antigenic peptide ₃₆₉YMDGTMSQV₃₇₇, which contains one of these two deamidated residues. We showed that the production of spliced peptide IYMDGTADFSF also required translation of tyrosinase into the ER (Fig. 1B), followed by proteasomal degradation in the cytosol (Fig. 3). We then evaluated the role of PNGase in the deamidation process leading to spliced peptide IYMDGTADFSF. We treated melanoma cells LB39-MEL with the caspase inhibitor z-VAD-fmk, which is also a potent inhibitor of PNGase (Altrich-VanLith et al., 2006; Misaghi et al., 2004), and tested them for recognition by the three CTL clones used previously. Treatment with z-VAD-fmk strongly inhibited presentation of peptide IYMDGTADFSF, and, to a lesser extent, peptide YMDGTMSQV (Fig. 4A). However, it did not affect presentation of peptide MLLAVLYCL, which does not contain a deamidated residue. Treatment of tumor cells with Q-VD-OPh, a general caspase inhibitor that does not block PNGase activity (Misaghi et al., 2004), had no effect on CTL recognition (Fig. 4A). This result indicated that PNGase plays a role in the processing of spliced peptide IYMDGTADFSF. To clarify the role of PNGase in the generation of this epitope, we transfected COS-7 cells with an HLA-A24-encoding construct and a construct encoding either full-length wild-type tyrosinase (Tyr N337 N371), or a full-length tyrosinase in which Asn at positions 337 and 371 were replaced by Asp (Tyr D337 D371). After transfection, we treated cells with z-VAD-fmk or Q-VD-OPh and tested their recognition by CTL CL62. In line with the results reported above with melanoma cells, z-VAD-fmk but not Q-VD-OPh, reduced recognition of cells transfected with Tyr N337 N371 (Fig. 4B, *upper part*). In contrast, z-VAD-fmk did not affect recognition of cells transfected with Tyr D337

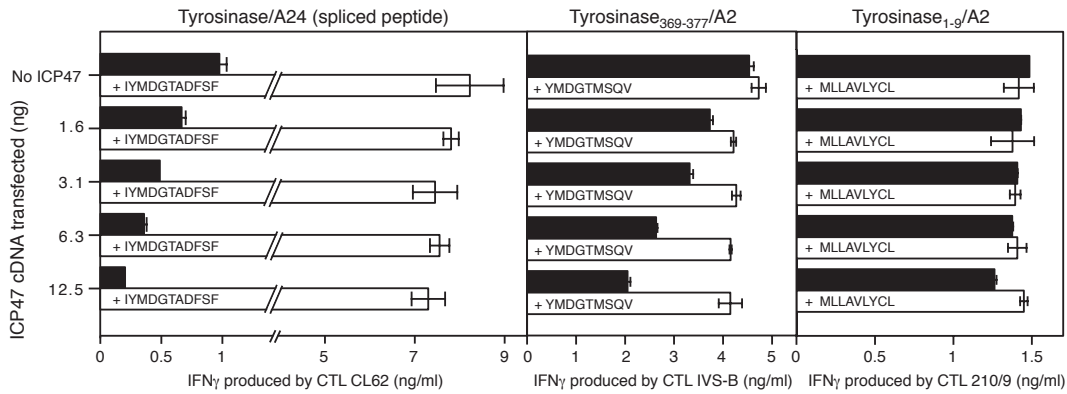


Figure 5. Role of TAP transporter in the processing of the tyrosinase spliced peptide.

COS-7 cells were transiently transfected with plasmids encoding tyrosinase and the appropriate HLA molecule (HLA-A24 or HLA-A2), together with the indicated amounts of a plasmid encoding ICP47, a herpes simplex viral protein that blocks TAP transport. Twenty four hours later, cells were tested for recognition by three different tyrosinase-specific CTL clones. CTL CL62 recognizes spliced peptide IYMDGTADFSF (left panel), CTL IVS-B recognizes the TAP-dependent peptide ₃₆₉YMDGTMSQV₃₇₇ (middle panel) and CTL 210/9 recognizes the TAP-independent peptide ₁MLLAVLYCL₉ (right panel). IFN γ production was measured after an overnight coculture. Where indicated (white bars), the corresponding antigenic peptide was pulsed onto transfected cells during one hour before addition of the CTL. Data shown are representative of at least two independent experiments. Error bars show standard deviations of triplicates.

D371 (Fig. 4B, *lower part*). We obtained similar results when we transfected a pair of shorter constructs, coding for tyrosinase₃₃₂₋₃₃₇ preceded by a signal peptide (SP-Tyr332-377) and containing no other *N*-glycosylation sites than N337 and N371 (Fig. 4C). Taken together, these results indicate that PNGase is responsible for the conversion of Asn residues into Asp during the processing of the spliced and doubly-deamidated peptide IYMDGTADFSF.

We next evaluated the role of TAP in the processing pathway of the tyrosinase spliced peptide using ICP47, a herpes simplex viral protein that blocks TAP transport (York et al., 1994). We transiently transfected COS-7 cells with plasmids encoding tyrosinase, ICP47 and the appropriate HLA molecule. Presentation of the spliced peptide IYMDGTADFSF to CTL CL62 was inhibited by ICP47 in a dose-dependent manner (Fig. 5). As expected, a similar inhibition was observed for the presentation of peptide YMDGTMSQV, while presentation of signal sequence-derived peptide MLLAVLYCL was not affected (Wölfel et al., 2000).

Discussion

In the present study, we identified a novel tumor antigenic peptide derived from tyrosinase and made of two spliced and reordered peptide fragments, each one containing an Asp residue resulting from Asn deamidation. Tyrosinase is a membrane-associated *N*-linked glycoprotein, which is synthesized, folded and glycosylated in the ER before being transported via the *trans*-Golgi network to melanosomes (Jimbow et al., 2000). Unfolded or misfolded tyrosinase molecules frequently accumulate in melanoma cells (Halaban et al., 2002) and represent an important source of MHC class I-restricted antigenic peptides recognized by melanoma specific T cells (Kang et al., 1995; Wölfel et al., 1994). These misfolded tyrosinase molecules are retained in the ER through prolonged interaction with lectin chaperones implicated in ER quality control and are further targeted for retrotranslocation to the cytosol via the ERAD pathway (Popescu et al., 2005; Svedine et al., 2004; Wang, Daniels, and Hebert, 2005). The study of the processing of the antigenic peptide tyrosinase₃₆₉₋₃₇₇ YMDGTMSQV demonstrated that, once in the cytosol, retrotranslocated tyrosinase molecules can be deglycosylated by PNGase and degraded by proteasomes (Altrich-VanLith et al., 2006). The antigenic peptide identified in the present study shares some amino-acid residues with the tyrosinase₃₆₉₋₃₇₇ epitope and appears to follow the same processing pathway. Indeed, we showed that its presentation to CD8⁺ T lymphocytes required translation of tyrosinase into the ER and was dependent on proteasome activity and TAP transport. We also observed that this epitope contains two Asp residues, one in each peptide fragment to be spliced, instead of the two Asn at positions 337 and 371 that were predicted by the sequence of the tyrosinase gene. This post-translational deamidation was already described for the Asn residue at position 371 during the processing of the tyrosinase₃₆₉₋₃₇₇ epitope (Skipper et al., 1996). It was demonstrated that deamidation of this Asn was partially due to its glycosylation in the ER and subsequent deglycosylation by PNGase in the cytosol (Altrich-VanLith et al., 2006). Here, by inhibiting PNGase activity with z-VAD-fmk in melanoma cells naturally expressing tyrosinase, we confirmed the involvement of this enzyme in the processing of the tyrosinase₃₆₉₋₃₇₇ epitope and showed that PNGase also contributes to the processing of the spliced antigenic peptide IYMDGTADFSF. We specified the role of PNGase by comparing the presentation of the spliced peptide between cells expressing wild-type tyrosinase and cells expressing a tyrosinase variant in which Asn at positions 337 and 371 were replaced by Asp. In both cases, transfected cells efficiently presented the antigenic peptide. However, we observed that inhibition of PNGase activity in cells expressing wt tyrosinase decreased the

presentation of the antigen, whereas no effect was observed in cells expressing the tyrosinase variant with the Asp residues. This observation confirmed that PNGase was responsible for the conversion of these Asn residues into Asp. This does not exclude, however, the existence of a second pathway, that would be responsible for deamidation of unglycosylated Asn residues present on some tyrosinase molecules, as suggested for the tyrosinase₃₆₉₋₃₇₇ peptide (Altrich-VanLith et al., 2006; Ostankovitch et al., 2009).

The presence of antigenic peptide IYMDGTADFSF under three different forms in the melanoma eluates was unexpected. These forms corresponded to three oxidation states of the methionine: a non-oxidized, a sulfoxide and a sulfone form (Fig. 2B). These three forms similarly activated CTL CL62 (Fig. S2), indicating that Met oxidation does not affect recognition of the spliced peptide by this CTL. However, this is not always the case. For example, the Met oxidation in a class I-restricted antigenic peptide derived from cytomegalovirus was shown to decrease the binding of the peptide-MHC complex to the TCR (Weiskopf et al., 2010). We wondered whether the oxidized forms of the spliced peptide were naturally produced in melanoma cells or were produced in vitro during the process of peptide elution. We observed that synthetic peptide IYMDGTADFSF spontaneously oxidized in vitro into the sulfoxide form. This oxidation is favored by drying. It was therefore not possible to determine whether the cells naturally presented the sulfoxide form of the peptide or not. Concerning the sulfone form of the peptide, we were surprised to detect its presence in the eluates because such a form was only obtained in vitro after treatment of the synthetic peptide with strong oxidative conditions. Additionally, intracellular proteins or peptides containing methionine sulfone were never described in the literature to our knowledge. To determine the origin of the sulfone form of the natural peptide, we exogenously loaded HLA-A24⁺ EBV B cells with a preparation of synthetic peptide IYMDGTADFSF containing the non-oxidized form with small amounts of the sulfoxide form. We then isolated the HLA class I-bound peptides present at the surface of these cells using identical experimental conditions as those used to isolate peptides from melanoma cells. This extract was separated on a HPLC column and the resulting fractions were tested in a CTL assay. Despite the absence of the sulfone form in the initial peptide preparation, three peaks of CTL activity were detected, corresponding to the three forms of the peptide (unpublished data). This indicated that the sulfone form of the peptide could be produced during the elution process. The factor responsible for this oxidation is unclear. One candidate is sodium azide, which was present in our lysis buffer and might cause oxidation of cysteine and methionine residues (Manneberg,

Lahm, and Fountoulakis, 1995). In conclusion, while showing that oxidation of the peptide can occur during the elution process, our results do not exclude the production of oxidized forms of the antigenic peptide by melanoma cells.

The role of the proteasome in the production of this newly identified spliced peptide was initially addressed by treating melanoma cells with proteasome inhibitor epoxomicin. We evaluated CTL recognition of these cells in a 5-hour degranulation assay and not by measuring IFN γ release by the CTLs after an overnight coculture. Indeed, when using IFN γ release as a read-out in such experiments, we observed an inhibition of the presentation of the three tyrosinase-derived antigenic peptides, including the proteasome-independent tyrosinase₁₋₉ peptide (unpublished data). This contrasted with the results published by Wölfel and colleagues, who tested CTL recognition of lactacystin-treated cells in a 4-hour ⁵¹Cr release assay and concluded that the proteasome was not involved in the production of this latter peptide (Wölfel et al., 2000). This difference could result from decreased levels of the tyrosinase protein, which were observed in cells after ten hours of proteasome inhibition (N. Parmentier, personal communication). Proteasome inhibition was found to induce cellular stress and reduce protein synthesis through phosphorylation of the alpha-subunit of the eukaryotic initiation factor-2 (Jiang and Wek, 2005). Because of this reduction of protein synthesis after proteasome inhibition, short CTL assays lasting 4 to 6 hours may be more appropriate than overnight CTL assays to evaluate the role of the proteasome in antigen processing.

The direct role of the proteasome in the production of spliced peptide IYMDGTADFSF was confirmed by in vitro experiments in which a precursor peptide was digested by purified 20S proteasomes. Standard proteasomes were able to produce the antigenic peptide IYMDGTADFSF whereas immunoproteasomes were not. Such differences between the two proteasome types to produce spliced peptides were already observed for the three known spliced peptides and resulted from a different capacity to produce the peptidic partners of the splicing reaction (Dalet et al., 2011).

The catalytic mechanism of the splicing reaction appears identical to that proposed for the other spliced peptides, involving a transpeptidation at the active N-terminal threonine of a catalytic β -subunit of the proteasome (Fig. 3D). Fragment IYMDGT is involved in the formation of an acyl-enzyme intermediate, linking by an ester bond the C terminus of this

fragment to the hydroxyl group of the side chain of an active threonine of the proteasome. During proteolysis, this intermediate is hydrolyzed and fragment IYMDGT is liberated. In the splicing reaction, the N-terminal amino group of fragment ADFSF competes with water molecules and performs the nucleophilic attack on the acyl-enzyme intermediate, leading to the creation of a new peptide bond and the production of spliced peptide IYMDGTADFSF. This transpeptidation model was validated by incubating various peptide pairs with purified proteasomes (Fig. 3E). Nevertheless, we also observed a weak activation of the CTL in response to digests obtained by incubating proteasomes with ADFSF and IYMDGT. According to the transpeptidation model, such digests should not contain the spliced antigenic peptide and activate the CTL, since the appropriate acyl-enzyme intermediate cannot form. When we studied the mechanism of production of the other spliced antigenic peptides, the incubation of the splicing partners together with proteasomes did not significantly stimulate the CTL (Dalet et al., 2010; Vigneron et al., 2004; Warren et al., 2006). This difference might be due to a higher sensitivity of CTL CL62 compared to the clones used in the previous studies. The weak activation of CTL CL62 did not result from a transpeptidation producing the shorter spliced peptides IYMDGADFSF or IYMDADFSF, since they are not recognized by CTL CL62 (Fig. S1). If the tyrosinase spliced peptide is indeed produced in this digest, this can only occur by a condensation reaction between ADFSF and IYMDGT within the proteasome, since the incubation of these two fragments in the absence of proteasomes did not lead to any detectable CTL activation (Fig. 3E). The mechanism of this reaction would involve therefore the interaction of peptide fragments with the proteasome, independently of proteolytic cleavage. A comparable phenomenon was described by studying the incorporation of ^{18}O into peptide fragments after protease digestion, a technique commonly employed for protein sequencing and quantitative proteomic studies (Angel and Orlando, 2006; Schnolzer, Jedrzejewski, and Lehmann, 1996). In these experiments, proteins were digested with endoproteases like trypsin in highly ^{18}O -enriched water, and since proteolysis proceeds via the incorporation of oxygen from water into the newly generated C-terminal carboxyl group of peptide fragments, proteolytic cleavage could be used to generate a set of ^{18}O -labeled peptides. However, in addition to incorporate one ^{18}O atom, peptide fragments were found to incorporate two atoms of ^{18}O in their C-terminal carboxyl group. This observation indicated that peptide fragments must continue to interact with the enzyme and can undergo repeated binding/hydrolysis cycles due to the reversibility of the reaction liberating the acyl-enzyme intermediate. In our case, this would imply that fragment IYMDGT is accepted as a pseudo-substrate by the proteasome to form a covalent ester intermediate with the activated hydroxyl

group of a catalytic threonine. This intermediate would be further hydrolyzed to give back fragment IYMDGT or, sometimes, aminolyzed by the N-terminal group of fragment ADFSF to give rise to the spliced peptide IYMDGTADFSF. If such a mechanism of reverse proteolysis really occurred in our in vitro experiment, it would contribute to a minor fraction of the spliced peptide produced (Fig. 3E). Remarkably, this condensation reaction seemed also to take place in living cells, since the electroporation of the splicing partners ADFSF and IYMDGT triggered a CTL activation, which is however about 6-fold weaker than the CTL response observed after electroporation of fragments ADFSF and IYMDGTMSQ (unpublished data).

Only four spliced peptides were identified to date as antigenic peptides recognized by specific T cells. Many other spliced peptides probably remain to be discovered, maybe with other relevant functions. Supporting this possibility, a recent study analyzing mass spectrometric data of in vitro proteasomal products with an algorithm that predicts all theoretically possible spliced peptides from a given molecule, revealed that the proteasome produces a substantial number of spliced peptides (Liepe et al., 2010). Therefore, despite the intrinsic low efficiency of the splicing reaction, it seems that the splicing activity of the proteasome is more important than initially thought, and is presumably facilitated by the catalytic and structural properties of this enzyme, as we previously suggested. The authors of this study proposed that peptide splicing is “an intrinsic additional catalytic property of the proteasome”. We have to keep in mind, however, that all proteases are theoretically able to catalyze peptide ligation. Peptide splicing has already been described in plants, and was found, in some cases, to be catalysed by vacuolar asparaginyl endopeptidase (Gillon et al., 2008; Min and Jones, 1994). Since this protease is involved in the production of antigenic peptides presented by MHC class II molecules (Watts et al., 2003), future studies may reveal the existence of spliced peptides that are not produced by the proteasome.

Materials and Methods

Cell lines and CTL clones

COS-7 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS. Melanoma cells LB33-MEL and LB39-MEL, HEK293-A24, renal carcinoma cells LB1047-RCC and LG2 EBV-transformed B cells were cultured in IMDM (Invitrogen) containing 10% FCS. HEK293 cells expressing the tetracycline repressor (T-Rex-293, Invitrogen) were stably transfected to express the three immunosubunits $\beta 1i$, $\beta 2i$ and $\beta 5i$ upon tetracycline exposure, as described before (Chapiro et al., 2006). These cells were called in this study 293-IP. Parental T-Rex-293 cells were used as cells expressing only standard proteasomes (293-SP). These two cell lines were cultured in DMEM medium containing 10% FCS and supplemented with D-glucose at 3,5 g/L. All culture media were supplemented with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), penicilline (100 U/ml) and streptomycin (100 μ g/ml).

TIL888 were generated by the culture of a fresh tumor sample in media containing 6000 UI/ml of human rIL2, as previously described (Rosenberg et al., 1988). The CTL clone 62 (CL62) was derived from TIL888 by carrying out limiting dilution as previously described at a concentration of 10 per well (Walter et al., 1995), with an estimated cloning efficiency of 1%. CTL SK29 clone IVS-B recognizes peptide tyrosinase₃₆₉₋₃₇₇ YMDGTMSQV and CTL LB24 clone 210/9 recognizes peptide tyrosinase₁₋₉ MLLAVLYCL, both presented by HLA-A2 (Wölfel et al., 1994). These CTLs were expanded as previously described (Murata, Warren, and Riddell, 2003) by stimulation every 14 d with 40 ng/ml anti-CD3 OKT3 monoclonal antibody (Ortho Biotech Products, Bridgewater, NJ), using irradiated (100 Gy) allogeneic PBMCs, in IMDM containing 10% human serum, human rIL2 (50 UI/ml), and IDO inhibitor 1-methyl-L-tryptophane (200 μ M) (Sigma-Aldrich, St. Louis, MO) and supplemented with amino acids and antibiotics as mentioned above.

Generation and analysis of tyrosinase plasmid constructs

Truncated tyrosinase constructs were generated by PCR from a full-length human tyrosinase cDNA, using specific primers. Following amplification, PCR products were cloned into pCDNA3/V5-His TOPO (Invitrogen). Point mutations N337D or N371D were introduced by carrying out a long PCR with primer 5'-AAAGCTGCCGATTTCAGCTT-3' or primer 5'-ATCTATATGGATGGAACAAT-3' respectively, using QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Corresponding non-mutated tyrosinase constructs

were used as templates to introduce one mutation and then a single mutant was used as template to introduce the second mutation. The truncated tyrosinase construct encoding amino acids 332 to 377 preceded by the signal peptide of interleukin-2 (Fig. 1B) was generated as follows: the construct 332-377 was amplified by PCR with a primer containing an additional sequence at the 5' end corresponding to an EcoRI site (5'-GAATTCGATGGATAAAGCTGCCAATTTC-3'), and a primer containing a NheI site at the 3' end (5'-GCTAGCTTGGCAGATCCCTATACCTG-3'). After amplification, the PCR product was digested with EcoRI and NheI and cloned into pFUSE-rFc2 (IL2ss) vector (Invivogen, San Diego, CA) which was also digested with EcoRI and NheI. To generate the construct encoding a tyrosinase variant with a deletion of amino acids 343 to 366 (Fig. 1D), the primers 5'-pATTTCTAAAGCTGAAATTGGC-3' and 5'-pCACATCTATATGAATGGAACA-3' were used in a PCR with full-length tyrosinase as template. The other construct, encoding a frameshifted product, contained an additional thymidine nucleotide (t) that was inserted between the codons encoding aa 342 and 366 and appeared to result from a PCR amplification error. The sequences of the coding region of all of the constructs generated were verified by sequencing with an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Transient transfections followed by CTL stimulation assay

COS-7 cells or HEK293 cells stably transfected with HLA-A24 (Fig. 1D) were plated in flat-bottom 96-well microplates 24 h before transfection. 293-SP and 293-IP cells were treated with tetracycline (1 µg/ml) at least 5 days before transfection (Fig. 3B). Cells were transfected in Optimem medium (Invitrogen) using Lipofectamine reagent (Invitrogen), with 50 ng of pCDNA3 plasmid coding for HLA-A24 or HLA-A2, and 250 ng (when not indicated) of pCDNA3 coding for the indicated tyrosinase construct. In figure 5, the indicated amounts of ICP47 cloned into PBJ1-Neo plasmid (gift from H.-G. Rammensee) were also co-transfected. Five hours after transfection, the medium was removed and transfected cells were cultured in their respective medium containing 10% FCS. When indicated, z-VAD-fmk or Q-VD-OPh (BioVision, Mountain view, CA) were added to the culture medium at a concentration of 50 µM (Fig. 4B and 4C). After an overnight incubation, the medium was carefully removed and CTLs were added in X-vivo 10 medium (BioWhittaker, Walkersville, MD). When indicated, synthetic peptides IYMDGTADFSF (1 µg/ml), or YMDGTMSQV (10 µg/ml), or MLLAVLYCL (10 µg/ml) were pulsed on transfected cells during 1 h before addition of the

corresponding CTL. After an overnight coculture, the supernatants were collected to measure their IFN γ content by ELISA (BioSource International, Camarillo, CA).

Isolation and fractionation of MHC-bound peptides present on melanoma cells

MHC class I-peptides complexes were immuno-purified using monoclonal antibody W6/32 after lysis of 6×10^9 LB33-MEL cells. Following elution with acid acetic 0.23 N pH 2.6, peptides were separated from HLA molecules in an Ultrafree-CL centrifugal 5kDa filter (Millipore, Billerica, MA). Peptides were further fractionated by HPLC after injection on a 3.9 x 150 mm Delta Pack C18 column (Waters, Milford, MA) at a flow rate of 0.9 ml/min with an elution gradient of 5-21% ACN/0.1% TFA (w/v) over 6 min followed by 21-28% ACN/0.1% TFA over 33 min. Fractions of 0.5 ml were collected, dried in centrifugal vacuum concentrator and resuspended in water. Half of each fraction was diluted in X-vivo 10 medium and loaded onto 20,000 HLA-A24⁺ EBV-B cells before addition of 15,000 CTL CL62 for the stimulation assay.

Proteasome inhibition in melanoma cells followed by CTL degranulation assay

Melanoma cells LB39-MEL, which naturally express tyrosinase, HLA-A2 and HLA-A24, were first treated at pH 3 for 1 min in a citrate buffer to elute MHC-bound peptides, and were neutralized with culture medium. Cells were then treated during 2 h at 37°C with 0.5 μ M epoxomicin (Sigma-Aldrich). They were washed and incubated during one more hour with 10 nM epoxomicin. At the same time, one half of the cells was divided into 3 parts and each part was pulsed during one hour with one of the following synthetic antigenic peptides: IYMDGTADFSF (2 μ g/ml), YMDGTMSQV (10 μ g/ml) and MLLAVLYCL (10 μ g/ml). After the peptide pulse, medium was changed and 150,000 LB39-MEL cells were incubated with 50,000 CTLs during 5 h, in the presence of FITC-labeled CD107a and CD107b Abs (BD Biosciences, San Jose, CA). Brefeldin A (Sigma-Aldrich) was added at 10 μ g/ml after one hour of incubation to prevent internalization of surface CD107 molecules. Cells were then stained with APC-labeled CD8 Ab (BD Biosciences) and finally with propidium iodide (Sigma-Aldrich) just before flow cytometry analysis on a FACSCalibur (BD Biosciences) to determine the proportion of CD8⁺ cells that have degranulated during the assay.

Peptide-N-glycanase inhibition in melanoma cells

LB39-MEL cells were first treated at pH 3 for 1 min in a citrate buffer to elute MHC-bound peptides, and were neutralized with culture medium. Cells were then treated during 5 h at

37°C with 50 μ M z-VAD-fmk or Q-VD-OPh. Cells were washed and resuspended in X-vivo 10 medium. One half of the cells was divided into 3 parts and each part was pulsed with one of these synthetic antigenic peptides: IYMDGTADFSF (2 μ g/ml), YMDGTMSQV (10 μ g/ml) and MLLAVLYCL (10 μ g/ml). After one hour, medium was changed and 40,000 LB39-MEL cells were incubated overnight with 15,000 CTLs CL62, or 30,000 CTLs IVS-B, or 20,000 CTLs 210/9. IFN γ release was determined by ELISA.

Peptide synthesis and recognition assay

Synthetic peptides were produced using Fmoc-solid phase chemistry and purified by reversed-phase HPLC. They were dissolved in DMSO at 20-30 mg/ml and were > 95 % pure. For CTL recognition assays, peptides were loaded onto 20,000 HLA-A24⁺ EBV-B cells for 30 min at 37°C and 15,000 CTLs CL62 were added. IFN γ release was determined by ELISA after an overnight incubation.

In vitro proteasome digestions

20S proteasomes were purified as previously described (Chapiro et al., 2006). Standard proteasomes were obtained from human erythrocytes while immunoproteasomes were purified from LCL-721 cells treated with IFN γ . Digestions were performed at 37°C by incubating 10 μ g (Fig. 3C) or 4 μ g (Fig. 3E) of each precursor peptide with 1 μ g of proteasomes in 20 μ l of Tris 10 mM pH 8. Digestions were stopped by addition of 2 μ l of 10% trifluoroacetic acid, lyophilized and resuspended in 20 μ l of water. One half (Fig. 3C) or one quarter (Fig. 3E) of each digest was diluted in 100 μ l of X-vivo 10 medium and loaded in duplicate wells containing 20,000 HLA-A24⁺ EBV-B cells. CTL CL62 (15,000 cells) was added 30 min later and IFN γ release was determined by ELISA after an overnight incubation.

Analysis by HPLC coupled to mass spectrometry

Fractions were analyzed by μ HPLC-MS/MS with a LTQ XL ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) fitted with a capillary HPLC (LC Packings, Sunnyvale, CA). The samples were separated by reverse-phase chromatography on an Alltech Prosphere 300 C18 (0.15/150 mm) column (Grace, Deerfield, IL) and eluted at a flow rate of 1.5 μ l/min with a 55 min linear gradient of acetonitrile in water (5–50%) containing 0.1% formic acid. Mass spectrometry analysis was performed on line with the mass spectrometer equipped with a microelectrospray probe and operated in positive mode with default parameters and active

automatic gain control. Mass spectra were acquired in a mode that alternated single MS scans (m/z 300-2000) with MS/MS scans using low energy collision-induced dissociation with relative collision energy of 35%.

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SUPPLEMENTARY MATERIAL

<u>Pool 1</u>	<u>Pool 2</u>	<u>Pool 3</u>	<u>Pool 4</u>	<u>Pool 5</u>
IYMDGTMSMDKAADFSF	IYMDGTMMDKAADFSF	IYMDGTMDKAADFSF	IYMDGMDKAADFSF	IYMDMDKAADFSF
IYMDGTMSDKAADFSF	IYMDGTMDKAADFSF	IYMDGTDKAADFSF	IYMDGDKAADFSF	IYMDDKAADFSF
IYMDGTMSKAADFSF	IYMDGTMKAADFSF	IYMDGTKAADFSF	IYMDGKAADFSF	IYMDKAADFSF
IYMDGTMSAADFSF	IYMDGTMAADFSF	IYMDGTAAADFSF	IYMDGAAADFSF	IYMDAADFSF
IYMDGTMSADFSF	IYMDGTMAADFSF	IYMDGTADFSF	IYMDGADFSF	IYMDADFSF
IYMDGTMSDFS	IYMDGTMDFSF	IYMDGTDFS	IYMDGDFS	IYMDDFS
<u>Pool 6</u>	<u>Pool 7</u>	<u>Pool 8</u>	<u>Pool 9</u>	<u>Pool 10</u>
IYMDGTMSMDKAADF	IYMDGTMMDKAADF	IYMDGTMDKAADF	IYMDGMDKAADF	IYMDMDKAADF
IYMDGTMSDKAADF	IYMDGTMDKAADF	IYMDGTDKAADF	IYMDGDKAADF	IYMDDKAADF
IYMDGTMSKAADF	IYMDGTMKAADF	IYMDGTKAADF	IYMDGKAADF	IYMDKAADF
IYMDGTMSAADF	IYMDGTMAADF	IYMDGTAADF	IYMDGAADF	IYMDAADF
IYMDGTMSADF	IYMDGTMAADF	IYMDGTADF	IYMDGADF	IYMDADF
IYMDGTMSDF	IYMDGTMDF	IYMDGTDF	IYMDGDF	IYMDDF
<u>Pool 11</u>	<u>Pool 12</u>	<u>Pool 13</u>	<u>Pool 14</u>	<u>Pool 15</u>
HIYMDGTMSMDKAADFSF	HIYMDGTMMDKAADFSF	HIYMDGTMDKAADFSF	HIYMDGMDKAADFSF	HIYMDMDKAADFSF
HIYMDGTMSDKAADFSF	HIYMDGTMDKAADFSF	HIYMDGTDKAADFSF	HIYMDGDKAADFSF	HIYMDDKAADFSF
HIYMDGTMSKAADFSF	HIYMDGTMKAADFSF	HIYMDGTKAADFSF	HIYMDGKAADFSF	HIYMDKAADFSF
HIYMDGTMSAADFSF	HIYMDGTMAADFSF	HIYMDGTAAADFSF	HIYMDGAAADFSF	HIYMDAADFSF
HIYMDGTMSADFSF	HIYMDGTMAADFSF	HIYMDGTADFSF	HIYMDGADFSF	HIYMDADFSF
HIYMDGTMSDFS	HIYMDGTMDFSF	HIYMDGTDFS	HIYMDGDFS	HIYMDDFS
<u>Pool 16</u>	<u>Pool 17</u>	<u>Pool 18</u>	<u>Pool 19</u>	<u>Pool 20</u>
HIYMDGTMSMDKAADF	HIYMDGTMMDKAADF	HIYMDGTMDKAADF	HIYMDGMDKAADF	HIYMDMDKAADF
HIYMDGTMSDKAADF	HIYMDGTMDKAADF	HIYMDGTDKAADF	HIYMDGDKAADF	HIYMDDKAADF
HIYMDGTMSKAADF	HIYMDGTMKAADF	HIYMDGTKAADF	HIYMDGKAADF	HIYMDKAADF
HIYMDGTMSAADF	HIYMDGTMAADF	HIYMDGTAADF	HIYMDGAADF	HIYMDAADF
HIYMDGTMSADF	HIYMDGTMAADF	HIYMDGTADF	HIYMDGADF	HIYMDADF
HIYMDGTMSDF	HIYMDGTMDF	HIYMDGTDF	HIYMDGDF	HIYMDDF
<u>Pool 21</u>	<u>Pool 22</u>	<u>Pool 23</u>	<u>Pool 24</u>	<u>Pool 25</u>
YMDGTMSMDKAADFSF	YMDGTMMDKAADFSF	YMDGTMDKAADFSF	YMDGMDKAADFSF	YMDMDKAADFSF
YMDGTMSDKAADFSF	YMDGTMDKAADFSF	YMDGTDKAADFSF	YMDGDKAADFSF	YMDDKAADFSF
YMDGTMSKAADFSF	YMDGTMKAADFSF	YMDGTKAADFSF	YMDGKAADFSF	YMDKAADFSF
YMDGTMSAADFSF	YMDGTMAADFSF	YMDGTAADFSF	YMDGAAADFSF	YMDAADFSF
YMDGTMSADFSF	YMDGTMAADFSF	YMDGTADFSF	YMDGADFSF	YMDADFSF
YMDGTMSDFS	YMDGTMDFSF	YMDGTDFS	YMDGDFS	YMDDFS
<u>Pool 26</u>	<u>Pool 27</u>	<u>Pool 28</u>	<u>Pool 29</u>	<u>Pool 30</u>
YMDGTMSMDKAADF	YMDGTMMDKAADF	YMDGTMDKAADF	YMDGMDKAADF	YMDMDKAADF
YMDGTMSDKAADF	YMDGTMDKAADF	YMDGTDKAADF	YMDGDKAADF	YMDDKAADF
YMDGTMSKAADF	YMDGTMKAADF	YMDGTKAADF	YMDGKAADF	YMDKAADF
YMDGTMSAADF	YMDGTMAADF	YMDGTAADF	YMDGAADF	YMDAADF
YMDGTMSADF	YMDGTMAADF	YMDGTADF	YMDGADF	YMDADF
YMDGTMSDF	YMDGTMDF	YMDGTDF	YMDGDF	YMDDF

Figure S1. List of the pools of synthetic peptides tested for CTL recognition.

Thirty pools (1-30) of 6 synthetic peptides corresponding to the reverse splicing of two non-contiguous tyrosinase fragments were prepared and tested for recognition by CTL CL62 after being loaded onto HLA-A24+ presenting cells. Positive pools (in red) were fractionated by HPLC and fractions were tested for CTL recognition (unpublished data). Peptides present in the positive fractions (in red) were synthesized, purified and tested for CTL recognition (Fig. 2A).

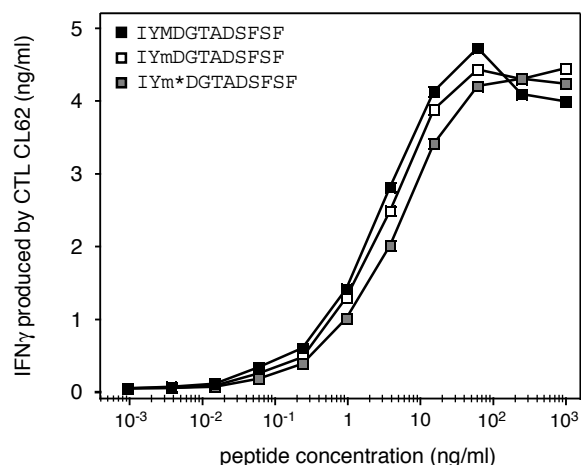


Figure S2. CTL recognition of oxidized forms of peptide IYMDGTADFSF.

The three forms of peptide IYMDGTADFSF, containing a non-oxidized methionine M (black symbols), a methionine sulfoxide m (white symbols) or a methionine sulfone m* (grey symbols) were purified, loaded onto HLA-A24+ EBV-B cells and tested for CTL recognition. IFN γ production was measured after an overnight incubation. Data shown are representative of two independent experiments performed in duplicate.

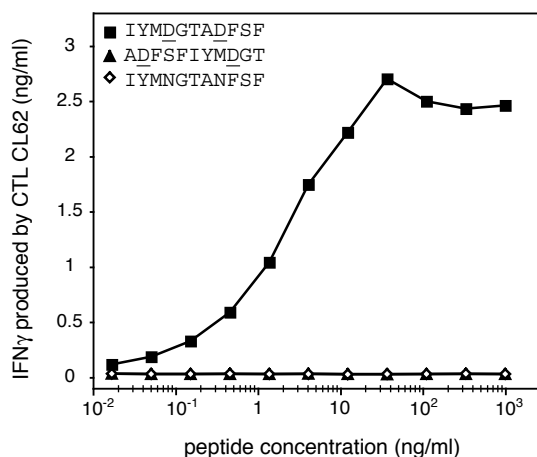


Figure S3. Peptide recognition by CTL CL62.

The indicated synthetic peptides, made of two non-contiguous fragments of tyrosinase, were loaded onto HLA-A24+ EBV-B cells and tested for recognition by CTL CL62. IFN γ production was measured after an overnight incubation. Data from one experiment performed in duplicate are shown.

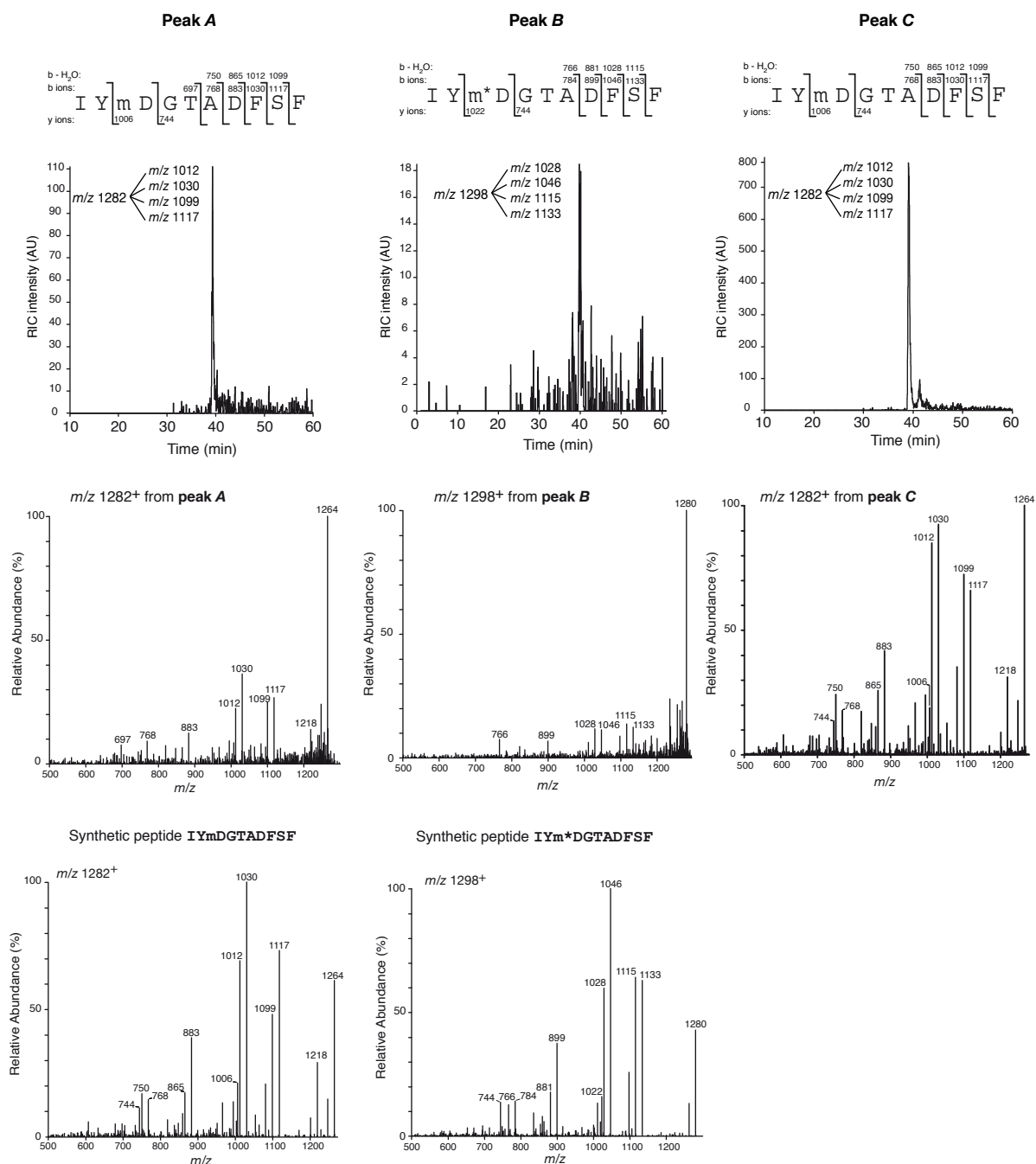


Figure S4. HPLC-MS/MS detection of the antigenic peptide in the three different peaks of CTL activity observed after fractionation of LB33-MEL eluates (see Fig. 2B).

Peak A comprises fractions 22.5-23.5; peak B comprises fractions 25.5 and 26; and peak C corresponds to fraction 32.5. Fractions corresponding to each peak of CTL activity were pooled and analysed by HPLC-MS/MS. The fragments that were detected are indicated above the peptide sequence for N-terminal b ions and their dehydrated forms, and below the sequence for C-terminal y ions. In the peptide sequence, m indicates a methionine sulfoxide, and m* a methionine sulfone. *Top panels*: reconstructed ion current (RIC) chromatograms representing the current measured for the four most characteristic ions of the antigenic peptide that are obtained after MS/MS analysis of ion with *m/z* 1282, which corresponds to singly charged ion IYmDGTADSF (peaks A and C), or ion with *m/z* 1298, which corresponds to singly charged ion IYm*DGTADSF (peak B). In each sample (peak A, B or C), the sum of these ion currents forms a peak whose retention time corresponds to the retention time observed for the synthetic peptides IYmDGTADSF and IYm*DGTADSF analysed in the same conditions (unpublished data). *Middle panels*: fragmentation spectra of ion with *m/z* 1282 detected in peak A (left) and C (right), and ion with *m/z* 1298 detected in peak B (middle). *Bottom panels*: fragmentation spectra of synthetic peptides IYmDGTADSF (left) and IYm*DGTADSF (right). Ions with *m/z* 1264 and *m/z* 1280 are the dehydrated derivatives of parental ions with *m/z* 1282 and *m/z* 1298, respectively. Ion with *m/z* 1218 corresponds to the loss of methanesulfenic acid from the methionine sulfoxide of parental ion 1282. Data shown are representative of two independent experiments for peak C, and one for peak A and B.

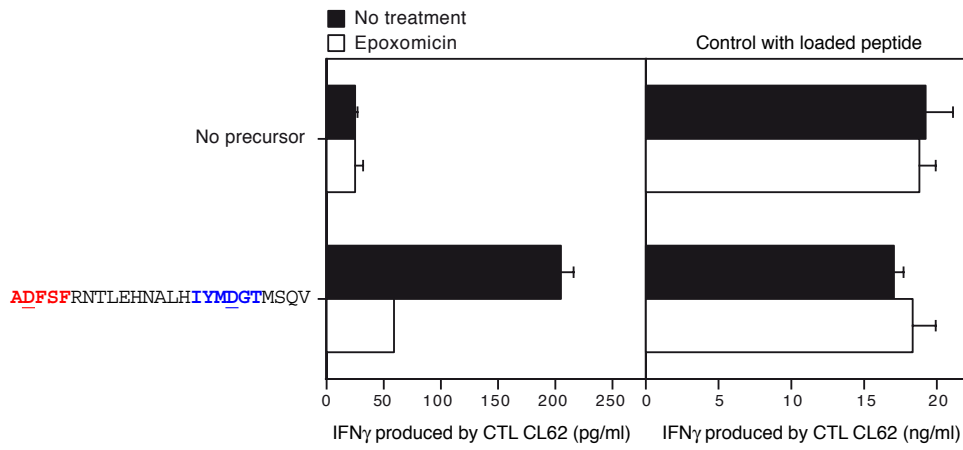


Figure S5. Proteasome involvement in the presentation of the tyrosinase spliced peptide by cells electroporated with a precursor peptide.

Renal carcinoma cells LB1047-RCC, expressing HLA-A24, were treated or not with 0.5 μ M epoxomicin during 1 h before electroporation of the indicated precursor peptide (200 μ M). After electroporation, cells were incubated at 37°C during 4 h with the precursor at a final concentration of 13 μ M, with or without 10 nM epoxomicin. For the CTL assay, 40,000 electroporated cells were incubated overnight with 15,000 CTL CL62 (left panel). For controls (right panel), the synthetic peptide IYMDGTADFSF was pulsed onto the electroporated cells during 30 min before the CTL assay. Data shown are representative of four independent experiments. Error bars show standard deviations of triplicates.

DISCUSSION

This work contributes to the recent findings on peptide splicing by the proteasome and provides further insights into this phenomenon.

First, we demonstrated that the transpeptidation model of peptide splicing by the proteasome also accounts for the production of the FGF-5 spliced antigenic peptide, despite the large distance that separates the splicing partners. This indicates that the proteasome is able to splice two peptide fragments even if they do not seem to be produced simultaneously in the catalytic chamber. In addition, we have observed that *trans*-splicing can occur as efficiently as *cis*-splicing when substrates are peptides, but not with full-length proteins.

By comparing the production of the three spliced antigenic peptides by the standard proteasome and the immunoproteasome, we observed that the gp100 and FGF-5 peptides are produced more efficiently by the standard proteasome, whereas the reversed spliced peptide derived from SP110 is produced better by the immunoproteasome. Moreover, the efficiency of production of a given spliced peptide appears to be directly linked to the production of the splicing partner fragments, and depends on a balance between cleavages liberating or destroying these fragments.

Furthermore, we have identified a new spliced antigenic peptide, derived from tyrosinase and presented by HLA-A24. The proteasome is responsible for the splicing reaction leading to the production of this antigen, which is composed of two fragments that are spliced together in the reverse order. An additional particularity of this antigenic peptide is that each fragment of the final epitope contains an aspartate residue instead of the asparagine residue encoded by the amino acid sequence of the tyrosinase gene. This post-translational modification appears to be catalysed by peptide-*N*-glycanase during deglycosylation of *N*-linked asparagine residues. The potential role of an additional protease in the processing of this peptide will be discussed in this section.

I) Implications of the differential processing observed for the spliced peptides between the standard proteasome and the immunoproteasome

The lack of processing of the FGF-5 and tyrosinase spliced peptides by the immunoproteasome, and, to a certain extent, the poor processing of the gp100 spliced peptide by the immunoproteasome could have implications for the use of these peptides for cancer

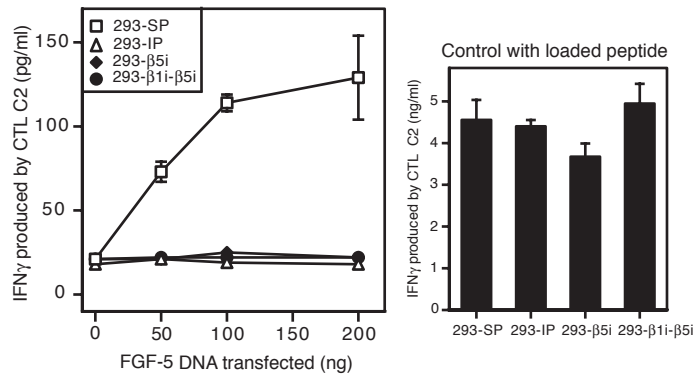
immunotherapy (**Articles 2 et 3**). It was shown that peptides that are poorly processed by the immunoproteasome are not efficiently presented by dendritic cells, which contain a larger proportion of immunoproteasomes (131, 262). To induce effective immune responses against such antigens, vaccine strategies should therefore bypass processing in dendritic cells (DCs) (260). This can be achieved by using peptides or minigene constructs encoding only the peptide, as opposed to protein or full-length constructs. This was confirmed in vivo using the HLA-A2-restricted Melan-A₂₆₋₃₅ peptide EAAGIGILTV, which is poorly processed by the immunoproteasome (338). Stronger Melan A-specific CTL responses were induced in HLA-A2 transgenic mice injected with minigene-containing retroviral constructs as compared to full-length constructs. This difference was abolished in β 1i-knockout mice, which did not have immunoproteasomes. These mice, as opposed to “wild-type” mice, developed strong responses to the full-length constructs, demonstrating that the immunoproteasome was responsible for the poor response of the “wild-type” mice. In line with this study, it was recently shown that reducing the expression of the three immunosubunits with siRNA in human DCs increases the level of standard proteasomes and leads to an altered repertoire of tumor antigenic peptides presented by these DCs (339). Interestingly, these « immunoproteasome-deficient » mature DCs triggered better antimelanoma immune responses than normal mature DCs in vitro after transfection with RNA encoding several melanoma differentiation antigens.

The poor processing of the three spliced peptides by the immunoproteasome may contribute to explain why CD8⁺ T cells recognizing these peptides, which derive from self proteins, are not deleted in the thymus by negative selection. Ectopic expression of tissue-specific antigens was indeed observed in the thymus, which also contains high amounts of immunoproteasomes (119, 340, 341).

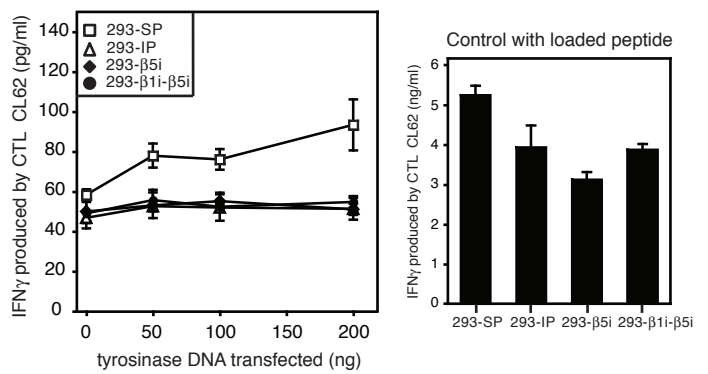
II) Processing of the spliced peptides by the intermediate proteasomes

The characterization in our laboratory of two intermediate proteasome subtypes, between the standard proteasome and the immunoproteasome, and their presence in normal tissues, as well as in tumor and dendritic cells, may have implications in the processing of antigenic peptides (131). The activity and functions of the different proteasome subtypes could be studied by

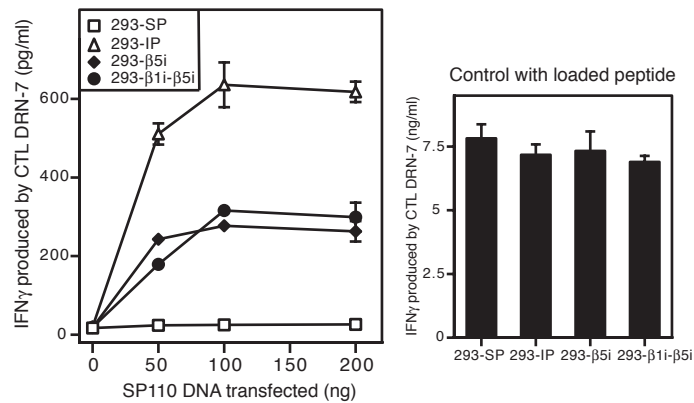
A Peptide FGF-5 NTYASPRFK



B Peptide tyrosinase IYMDGTADFSF



C Peptide SP110 SLPRGTSTPK



D Peptide gp100 RTKQLYPEW

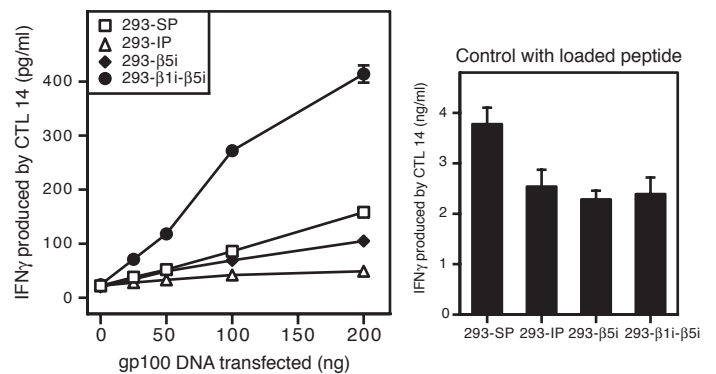


Figure 24. Processing of the spliced antigenic peptides by HEK293-EBNA cells containing different proteasome subtypes. Cells were produced as described in [131] and were transiently transfected with the indicated amounts of plasmid encoding parental protein and with 50 ng of plasmid encoding appropriate HLA molecule when necessary (HEK293-EBNA cells naturally express HLA-A3). Specific CTL was added 24 h after transfection and IFN γ was measured by ELISA after an overnight co-culture (*left panels*). As positive control, cells were pulsed with the corresponding antigenic peptide (1 μ g/ml) just before the CTL assay (*right panels*).

producing cells that are composed exclusively of immunoproteasomes, or intermediate $\beta 5i$ -proteasomes, or $\beta 1i$ - $\beta 5i$ -proteasomes (the corresponding parental cell line only expresses standard proteasomes). The role of the intermediate proteasomes in the processing of the spliced peptides was addressed by transiently transfecting these cell lines with plasmids encoding the corresponding parental protein and the appropriate HLA. The ability of transfected cells to stimulate specific CTLs was then assessed by measuring the production of IFN γ after an overnight co-culture. No major change was observed regarding the presentation of the spliced peptides derived from FGF-5, SP110 and tyrosinase, compared to the results obtained in articles 2 and 3, meaning that the intermediate proteasomes do not play a specific role in the production of these epitopes. The FGF-5 and tyrosinase spliced peptides were exclusively presented by cells expressing standard proteasomes (**Figures 24A, 24B**). The two intermediate proteasomes behaved like the immunoproteasome and did not produce these two spliced peptides. In contrast, the SP110 spliced peptide was efficiently presented by cells containing immunoproteasomes, but not by cells containing standard proteasomes (**Figure 24C**). Here, cells containing $\beta 5i$ -proteasomes or $\beta 1i$ - $\beta 5i$ -proteasomes presented equally the SP110 antigenic peptide, albeit less efficiently than immunoproteasome-containing cells. Concerning the gp100 spliced peptide, we observed, in article 2, a more efficient presentation in cells expressing standard proteasomes, than in cells expressing immunoproteasomes. However, when including cells with intermediate proteasomes, a more efficient presentation was observed in cells containing $\beta 1i$ - $\beta 5i$ -proteasomes (**Figure 24D**). Indeed, a ~3-fold higher CTL activation was triggered by these cells after gp100 and HLA-A32 transfection, compared to cells expressing standard proteasomes. This peptide was poorly presented by immunoproteasome-containing cells, and only weakly by cells containing $\beta 5i$ -proteasomes. From these observations, it seems that $\beta 2i$ is deleterious for the production of the gp100 spliced peptide and, on the contrary, the presence of $\beta 1i$ seems to favor its production. Together, these results complete the data obtained in article 2 by including all the characterized proteasome subtypes. Further experiments are required, however, to determine the mechanisms responsible for the differences observed in the production of the gp100 spliced peptide. Digests of a precursor peptide with purified intermediate proteasomes and the analysis of the cleavage products would certainly provide some interesting clues regarding this differential processing.

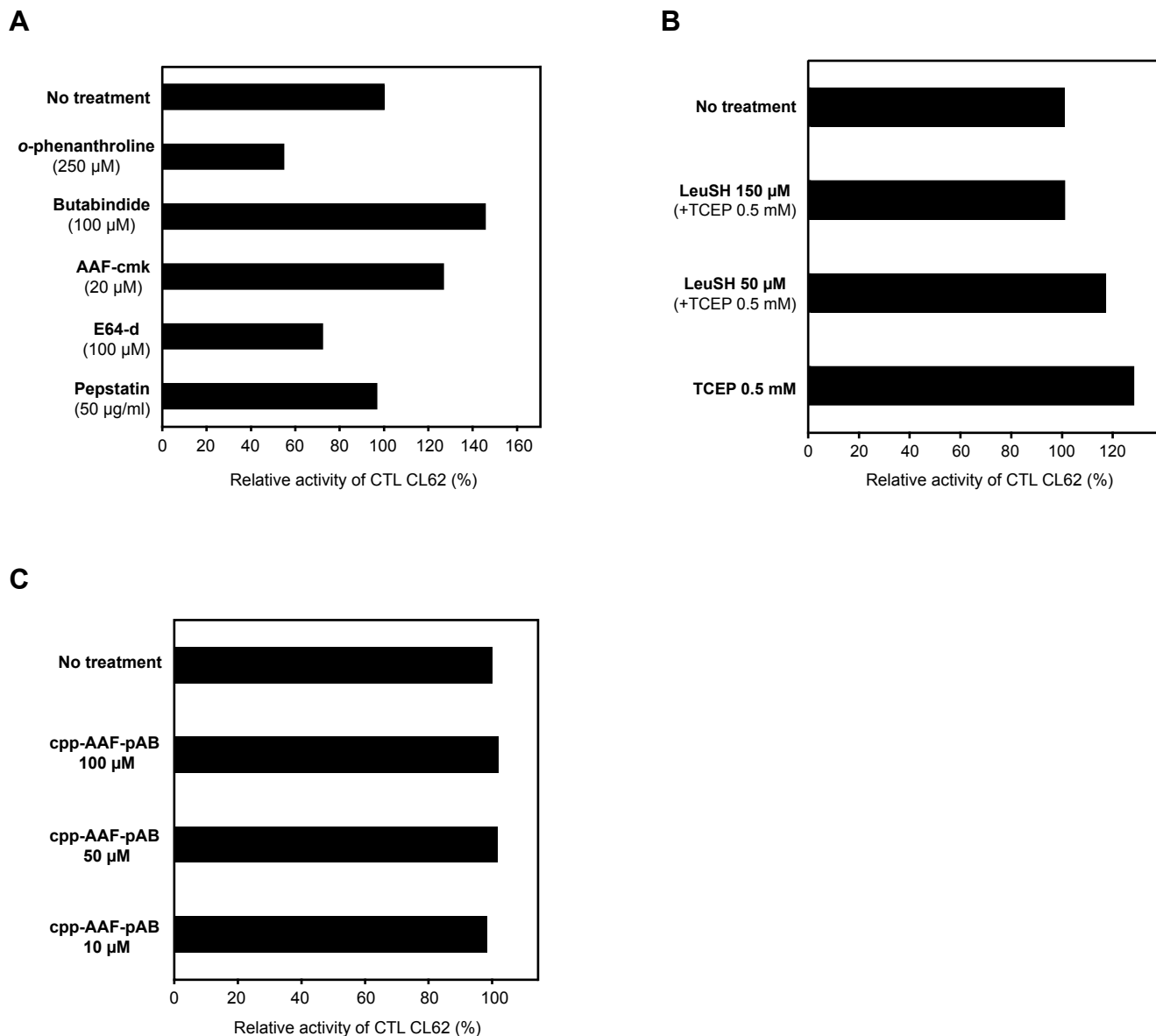


Figure 25. Treatment of melanoma cells with various protease inhibitors and effect on CTL recognition.

Melanoma cells LB33-MEL (*A*) and LB39-MEL (*B* and *C*) naturally express tyrosinase and HLA-A24. These cells were first incubated during 1 min 30 s in citrate buffer pH 3 to elute MHC-bound peptides, and were neutralized with culture medium. In *A* and *C*, cells were then treated during 5 h with the indicated compounds. In *B*, cells were treated during 20 h with Leucinethiol, whose sulfhydryl reactive group is maintained in a reduced active form with an antioxydant, here TCEP. All treatments were realized in culture medium without FCS to minimize compound degradation. After treatment, cells were washed and tested for CTL recognition. IFN γ production by CTL was measured by ELISA after an overnight incubation. As controls, half of the cells were pulsed with the synthetic epitope IYMDGTADFSF (1 μ g/ml) during one hour before addition of the CTL. Results show the relative CTL activation that was calculated first as a proportion of the activity measured with the synthetic peptide control. This proportion was then related to that observed without treatment, which was taken as 100%. Actual values (not shown) are determined from triplicates.

III) Potential role of a second protease in the processing of the tyrosinase spliced peptide

In addition to the proteasome, which is responsible for the splicing reaction leading to the tyrosinase spliced peptide IYMDGTADFSF, some experimental results strongly suggested that another protease was involved in the processing of this antigenic peptide. Indeed, treatment of melanoma cells with various protease inhibitors indicated that ortho-phenanthroline (*o*-phe), which is a general metalloprotease inhibitor, altered CTL recognition of treated cells (**Figure 25A**). On the contrary, treatment with butabindide, which is a TPPII-specific inhibitor, or with AAF-cmk, which is known to inhibit TPPII but also other proteases implicated in antigen processing, such as puromycin-sensitive aminopeptidase and bleomycine hydrolase (286), enhanced CTL recognition of melanoma cells, suggesting that TPPII may contribute to destroy the tyrosinase spliced peptide. Treatment of melanoma cells with pepstatin A, an inhibitor of aspartic proteases, had no effect on the presentation of the epitope, whereas treatment with the cysteine protease inhibitor E-64d induced a weak, but significant, decrease in peptide presentation. We supposed that this decrease might be due to potential inhibition of PNGase activity, which is responsible during processing of the tyrosinase spliced peptide, for the conversion of the two asparagine residues into aspartates (**Article 3**). PNGase is known to be inhibited by the caspase inhibitor z-VAD-fmk via the formation of a thioether adduct with the cysteine residue of the catalytic triad Cys-His-Asp (342). Given the identical mechanism of cysteine protease inhibition by fluoromethylketones (such as z-VAD-fmk) and by E-64d, which both involve the formation of a covalent thioether bond with the sulfhydryl group of the cysteine residue present in the enzyme active site, it might be possible that E-64d impaired the production of the tyrosinase peptide by inhibiting PNGase. This hypothesis could be easily verified by analysing the effect of E-64d on the deglycosylation activity of a recombinant PNGase. However, a potential role of a cysteine protease in the processing of this spliced peptide cannot be totally excluded.

To go further, we focused on the effect of *o*-phe on the presentation of the epitope and we treated cells with more selective metallopeptidase inhibitors. No effect was observed on CTL recognition of melanoma cells treated with leucinethiol or cpp-AAF-pAB, which are known to inhibit ERAAP and TOP, respectively (**Figures 25B, 25C**). Whether these compounds were indeed active in our experiments should be controlled on cell extracts with appropriate fluorogenic substrates. Other metallopeptidase inhibitors were tested, such as bestatin,

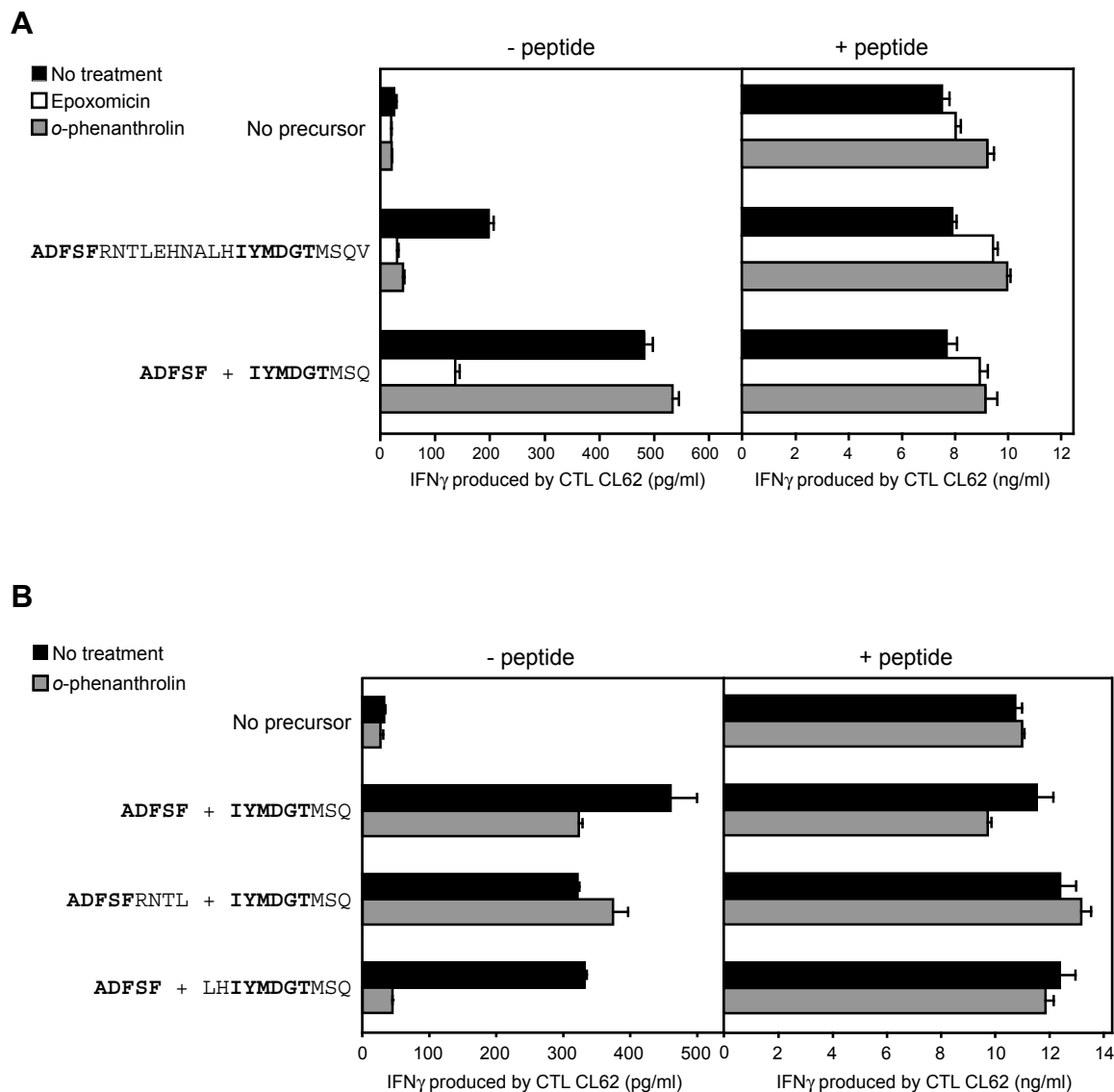


Figure 26. Electroporation of precursor peptides in cells treated with protease inhibitors, followed by CTL recognition. HLA-A24⁺ renal carcinoma cells LB1047-RCC were treated or not with 0.5 μ M epoxomicin (*A*) or 250 μ M *o*-phenanthroline (*A* and *B*) during 1 h before electroporation of the indicated peptides (200 μ M). After electroporation, cells were incubated at 37°C during 4 h in the presence of the peptides at a final concentration of 13 μ M, with or without 20 nM epoxomicin (*A*) or 250 μ M *o*-phenanthroline (*A* and *B*). Cells were then washed and incubated overnight with the CTL (*left panels*). IFN γ production was measured by ELISA in supernatants of culture. As controls, cells were pulsed with the synthetic peptide IYMDGTADFSF (1 μ g/ml) during 1 h before addition of the CTL (*right panels*). Error bars show standard deviations of triplicates.

amastatin and phosphoramidon but, despite their absence of toxicity, no significant effect on the presentation of the tyrosinase spliced peptide was observed.

If an additional protease, beside the proteasome, is indeed involved in the processing of the spliced peptide derived from tyrosinase, we hypothesized that this might be responsible for a post-proteasomal N-terminal trimming. Although the proteasome was found to be able to produce in vitro the naturally-occurring epitope from a precursor peptide, albeit with a very low efficiency (**Article 3**), splicing may occur in cells between fragment ADFSF and a N-extended peptide containing IYMDGT, so that the proteasome would produce a N-extended form of the reordered spliced peptide IYMDGTADFSF. This precursor could be further trimmed by a metallo-aminopeptidase to give rise to the final antigenic peptide. To support this hypothesis, we observed that the presentation of the spliced peptide was impaired by *o*-phe treatment of cells that had been electroporated with the 25-mer precursor peptide **ADFSFRNTLEHNALHIYMDGTMSQV** (**Figure 26A**). In contrast, no effect of the metalloprotease inhibitor was observed when cells were electroporated with both peptides **ADFSF** and **IYMDGTMSQ**. Notably, the treatment of cells with epoxomicin impaired the presentation of the antigenic peptide in both cases, indicating that the proteasome is indeed responsible for the splicing reaction. To determine which cleavage in the 25-mer precursor peptide was dependent on an *o*-phe-sensitive activity, cells were treated or not with *o*-phe and electroporated with various peptides combined in a pairwise manner. Presentation of the antigenic peptide was not impaired by *o*-phe treatment when peptides **ADFSFRNTL** and **IYMDGTMSQ** were electroporated simultaneously (**Figure 26B**). However, cells electroporated with **ADFSF** and **LHIYMDGTMSQ** were no longer recognized by the CTL after *o*-phe treatment. These results suggest therefore that the proteasome generates a N-extended spliced peptide, which may be further trimmed by a metallo-aminopeptidase to produce the mature spliced antigenic peptide. It should be noted that the reproducibility of the results presented here has to be confirmed.

Interestingly, a study has detailed the involvement of several proteases in the N-terminal trimming of the HLA-A2 restricted tyrosinase₃₆₉₋₃₇₇ peptide YMN(D)GTMSQV, providing some clues about the potential N-terminal trimming of the tyrosinase spliced peptide, which shares several residues with this other tyrosinase-derived epitope. The use of siRNA indicated that ERAP1 contributed to the processing of the tyrosinase₃₆₉₋₃₇₇ peptide (343). However, no effect on the presentation of the epitope was observed after using siRNA for bleomycin hydrolase, and an increased presentation was detected after transfecting siRNA for leucine

aminopeptidase, TPPII, TOP and puromycin-sensitive aminopeptidase, meaning that these proteases may contribute to destroy the antigen. Furthermore, in vitro degradation by ERAP1 of a precursor peptide N-terminally-extended with six residues indicated that this protease was indeed able to produce the N terminus of the tyrosinase₃₆₉₋₃₇₇ peptide and, interestingly, could also produce a precursor extended with a single residue (IYMNGTMSQV). This suggests therefore that ERAP1 may also be able to produce the N terminus of the spliced peptide IYMDGTADFSF.

Collectively, these observations support a potential N-terminal trimming by a metallopeptidase during the processing of the spliced peptide IYMDGTADFSF. This implies that the proteasome would produce an N-extended form of the spliced antigenic peptide, which would be further trimmed to the correct size. According to the study on the tyrosinase₃₆₉₋₃₇₇ peptide, ERAP1 could be an appropriate candidate in our case. However, the absence of decrease in the presentation of the epitope after leucinethiol treatment does not support this hypothesis. Further experiments are needed therefore to characterize the protease potentially implicated in this processing. As a first approach, we could transfect melanoma cells expressing the antigenic peptide with siRNAs targeting metalloproteases implicated in antigen processing, such as LAP, PSA, ERAP1, ERAP2 or IDE, and evaluate the effect of the siRNA on the CTL recognition of the cells. If these experiments provide information concerning the protease involved, we should then be able to perform in vitro digestions of a precursor peptide with purified proteasomes together with a purified or recombinant form of this protease.

IV) Catalytic mechanism of peptide splicing by the proteasome

Through the three studies presented in this work, we confirmed the catalytic mechanism of the splicing reaction proposed previously, which is a transpeptidation taking place in the catalytic chamber of the proteasome, at the active N-terminal threonine of a catalytic β -subunit. The splicing process occurs during the cleavage of peptide bonds, which involves the formation of an acyl-enzyme intermediate transiently linking the N-terminal peptide fragment to the proteasome subunit. During normal proteolysis, this intermediate is rapidly hydrolysed and the fragment is liberated. In the splicing reaction, the N terminus of another peptide

fragment competes with water molecules and performs a nucleophilic attack of the acyl-enzyme intermediate, resulting in the formation of a new peptide bond by transpeptidation. We previously proposed that this reaction was presumably favored in the confined catalytic chamber of the proteasome, which prevents the rapid diffusion of the peptide fragments that are generated, hence contributing to enhance their relative concentration. Here, the finding that the FGF-5 spliced peptide is produced by transpeptidation within the proteasome despite the more important distance between the splicing partners is consistent with this model, and demonstrates that splicing can occur between fragments of a given protein that are not produced simultaneously (**Article 1**). By shortening the distance between the FGF-5 splicing partners, we observed that the efficiency of peptide splicing is increased, presumably because the probability of interaction of the two relevant fragments in the catalytic chamber is enhanced. In the same time, these results support the model in which proteasomes degrade proteins in a processive manner (89, 90). The comparison of the production of a given spliced peptide between the standard proteasome and the immunoproteasome also supports the transpeptidation model of splicing by showing that the efficiency of production of a spliced peptide is directly linked to the capacity to produce the splicing partners (**Article 2**). This indicates that splicing is not intrinsically favored by specific structural features of a given proteasome type, but instead depends on the occurrence of cleavages at appropriate sites to produce the fragments to splice. Consistent with this observation and with the broad specificity of proteasomal cleavages, no substrate sequence specificity could be deduced from the four examples of spliced peptides described to date. These findings therefore imply that splicing could occur in theory between all fragments that are generated by the proteasome. Even though the literature on peptide splicing by the proteasome is very poor, a method combining computational and experimental approaches together with mass spectrometry (SpliceMet) was recently developed to facilitate the identification of proteasome-generated spliced peptides (344). By applying this method to *in vitro* digestions of synthetic polypeptides by purified 20S proteasomes, the authors could detect several different spliced peptides by mass spectrometry. For example, nine spliced peptides were detected from the digestion of a 23-mer gp100 peptide encompassing the fragments of the spliced antigenic peptide RTKQLYPEW. Several spliced peptides were also detected after the digestion of polypeptides derived from other proteins. They concluded that the proteasome represents a molecular machine that facilitates the production of spliced peptides from its own cleavage products. Similarly to our results, these findings support the notion that peptide splicing is not dependent on particular sequence motifs, but could instead occur at any cleavage site.

However, even though several distinct spliced peptides could be detected after proteasomal processing of a polypeptide, their number remains limited, presumably due to the intrinsic low efficiency of the reaction.

What can therefore favor the splicing of two given peptide fragments among many others? According to the catalytic mechanism of peptide bond cleavage, together with crystallographic and modelisation studies of the inner structure of the proteasome, it was proposed that the splicing reaction could be influenced by interactions between the peptide substrates and the substrate-binding sites (345). Peptide splicing would first depend on the stability of the acyl-enzyme, which is determined by specific interactions between side chains of the substrate residues and residues involved in the formation of the non-primed substrate-specificity channel. The acyl-enzyme should be stabilised at the active site long enough to allow a peptide fragment located at the primed specificity pockets to perform the nucleophilic attack on the carbonyl carbon atom of the acyl-enzyme, before a water molecule does so. Competition among various peptides for binding to the primed substrate-specificity channel would also determine the peptide to be spliced. The peptide fragment attacking the acyl-enzyme should be correctly stabilised and pre-oriented at the primed specificity pockets to allow the splicing reaction.

The proteasome and asparaginyl endopeptidase (AEP) are the two enzymes known to catalyse peptide splicing *in vivo*, the first one in humans and the second one in plants. In both cases, the catalytic mechanism of the reaction appears to be a transpeptidation involving an acyl-enzyme intermediate. In addition, proximity effects of the splicing partners seem to facilitate the splicing reaction. They can be mediated either by features inherent to the substrate (i.e. the primary or tertiary structure), or by intrinsic structural features of the enzyme (i.e. the confined catalytic chamber of the proteasome). Another common point concerns the energy required to form the new peptide bond. This energy is recovered from one of the bonds cleaved by the enzyme, so that the reaction can occur in the absence of ATP. Moreover, the affinity of the substrate for the specific binding pockets of the enzyme is thought, in both cases, to favor the splicing reaction.

Even though the transpeptidation model of peptide splicing by the proteasome seems to be accepted now, the existence of another mechanism contributing, albeit weakly, to peptide splicing within the proteasome was raised during the study of the tyrosinase spliced peptide IYMDGTADFSF (**Article 3**). A weak CTL activation could be triggered after incubating the

splicing partners ADFSF and IYMDGT with purified proteasomes, and also after introducing these two peptide fragments in living cells by electroporation. If we admit that the CTL is activated by the production of the antigenic peptide, this must be therefore produced by condensation of the two peptide fragments. This condensation would take place within the proteasome and would involve a catalytic β -subunit, since CTL activation was abolished in the absence of the proteasome or after its chemical inhibition. Proteases are defined as catalysts in chemistry and by definition, they alter the rate in which the thermodynamic equilibrium of a reaction is reached, but do not change the overall equilibrium between starting materials and proteolytic products (346). This implies that these enzymes can work reversibly in both directions of the reaction, being able therefore to catalyse peptide bond synthesis. This phenomenon is known as reverse proteolysis and is energetically unfavorable. However, various strategies can be used in vitro to promote peptide ligation, such as product removal by precipitation, the use of a large molar excess of one of the substrates, or the use of water-miscible organic co-solvents (327). Under physiological conditions, the equilibrium of the reaction largely favors peptide hydrolysis while reverse proteolysis is thought to be negligible. However, in some cases, molecular crowding was shown to promote peptide ligation, suggesting that in cells, proteases might also catalyse peptide bond synthesis. It is therefore possible that peptide ligation by reverse proteolysis would be facilitated in the catalytic chamber of the proteasome, due to the confinement of the substrates. The elevated, non-physiological concentrations of synthetic peptides used in our experiments may also favor reverse proteolysis, which is probably an extremely rare phenomenon in vivo.

V) *Cis*-splicing versus *trans*-splicing

While studying the production mechanism of the FGF-5 spliced antigenic peptide, we tested the possibility of splicing between two distinct substrates, which is named *trans*-splicing (**Article 1**). In contrast, splicing of peptide fragments coming from a single substrate is called *cis*-splicing. We observed that *trans*-splicing can be as efficient as *cis*-splicing when polypeptides are used as substrates. A substantial number of spliced peptides produced by *trans*-splicing were also identified using SpliceMet when precursor peptides from various origins were processed in vitro by purified proteasomes (344). However, the situation is very different in cells when substrates are full-length proteins, and *trans*-splicing in this case

appears to be far less efficient than *cis*-splicing. We proposed that this difference might result from an unequal probability, due to steric hindrance, that two proteins gain access simultaneously into the same proteasome particle, compared to two polypeptides. A single proteasome particle was found to be able to accommodate and process two or more protein substrates simultaneously (99, 347, 348). Nevertheless, the occurrence of this phenomenon for two given proteins might be too scarce to allow sufficient production of a given spliced peptide that would be relevant for antigen presentation and CTL recognition. This does not exclude the potential existence of spliced peptides that might be produced stochastically in cells by *trans*-splicing between two random proteins. However, the extremely low efficiency of this process should prevent such peptides from exerting any relevant biological function.

VI) General conclusion on the splicing of antigenic peptides

The major consequence of peptide splicing by the proteasome is to increase the diversity and the repertoire of antigenic peptides. By amplifying the diversity of peptides presented by MHC class I molecules, peptide splicing directly increases the chance that one or more of these epitopes are recognized by CD8⁺ T cells. However, the extent to which peptide splicing increases this diversity remains to be established. The transpeptidation model of splicing, which does not seem to depend on particular motif, but rather on cleavage preferences by the proteasome, predicts a combinatorial number of theoretical spliced peptides (345). However, because of the low efficiency of the reaction, this number must probably be reduced. So, does splicing remain a stochastic side-reaction or does it represent a new proteasome function, as was proposed by the group who developed SpliceMet? This question will remain difficult to answer, because peptide splicing is a silent post-translational modification, leaving no “mark” on a peptide or a protein. Even though SpliceMet can be a useful tool to detect spliced peptides *in vitro*, detection of such peptides *in vivo* will still be complicated.

To date, only two proteases were found to catalyse peptide splicing *in vivo*: AEP in plants and proteasome in humans. In both cases, ligation of peptide fragments appears to be favored by proximity effects or substrate confinement, and by affinity of the substrates for the specific binding pockets of the enzyme, conditions that can be met by other proteases *in vivo*. For example, TPPII and bleomycin hydrolase, whose catalytic centres are compartmentalized, could be good candidates for peptide splicing events. In this respect, bleomycin hydrolase was

found to catalyse peptide ligation in vitro (349). The transpeptidation model of peptide splicing is difficult to envisage, however, for metallo- and aspartic proteases, due to the absence of covalent acyl-enzyme intermediate in their catalytic mechanism. This does not exclude the existence of a different mechanism of peptide splicing which could be mediated by enzymes of these families. Furthermore, the presence of AEP in the lysosomes of some antigen-presenting cells and its role in the processing of MHC class II antigens (350), could contribute to the existence of spliced peptides presented by MHC class II molecules. Spliced peptides with non-immune functions, such as neuropeptides, might also be discovered in the future.

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