

Peptide splicing by the proteasome

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The proteasome is the major protease responsible for the production of antigenic peptides recognized by CD8⁺ cytolytic T cells (CTL). These peptides, generally 8–10 amino acids long, are presented at the cell surface by major histocompatibility complex (MHC) class I molecules. Originally, these peptides were believed to be solely derived from linear fragments of proteins, but this concept was challenged several years ago by the isolation of anti-tumor CTL that recognized spliced peptides, *i.e.* peptides composed of fragments distant in the parental protein. The splicing process was shown to occur in the proteasome through a transpeptidation reaction involving an acyl-enzyme intermediate. Here, we review the steps that led to the discovery of spliced peptides as well as the recent advances that uncover the unexpected importance of spliced peptides in the composition of the MHC class I repertoire.

The immune system constantly monitors cellular integrity to restrain tumor development or viral infections. CD8⁺ cytolytic T lymphocytes (CTL)⁴ are essential players in this process, because of their ability to recognize and destroy cells expressing tumoral or viral proteins. CTL indeed recognize peptides derived from these proteins and displayed at the cell surface by major histocompatibility complex (MHC) class I molecules. Peptides recognized by CTL are produced in the cytosol by the proteasome, a large protease complex responsible for the bulk of cellular protein degradation (1). A fraction of the peptides released by the proteasome is thus transferred into the lumen of the ER by a specialized transporter called TAP (transporter associated with antigen processing) (2), where they can be further trimmed by ER-resident amino peptidases (ERAP1 and ERAP2) (3–5) and loaded onto MHC class I molecules. Stable MHC–peptide complexes then exit the ER and migrate through the secretory pathway to reach the cell surface, where they will be displayed for CTL recognition (6). For a number of years, antigenic peptides recognized by CTL were believed to only

derive from linear fragments of cellular proteins. This concept was challenged by the identification of several antigenic peptides, composed of two peptide fragments that were originally distant in the parental protein but are assembled together by the creation of a new peptide bond (7–13). This process, called peptide splicing, was shown to take place in the proteasome (8–10). In this minireview, we will recapitulate the discovery of this phenomenon and the recent developments that highlight the unforeseen importance of peptide splicing in the establishment of the MHC class I peptide repertoire.

The proteasome

The proteasome is the central component of the ubiquitin–proteasome system, which is the major ATP-dependent protein degradation system in cells. The 20S proteasome is organized as a cylindrical particle containing four stacked heptameric rings, which delimit the catalytic chamber where the proteins are degraded (Fig. 1A) (14). The two inner rings are made of seven β subunits (β 1– β 7), and the two outer rings are made of seven α subunits (α 1– α 7). Three β subunits (β 1, β 2, and β 5) are catalytically active in vertebrate proteasomes and will initiate the cleavage of the peptide bonds in proteins. Entry of proteins into the catalytic chamber is tightly regulated. Indeed, the narrow pore formed by the α ring is further obstructed by the N-terminal tails of these α subunits (14–16); this prohibits the entry of folded proteins into the proteasome chamber and thereby protects cells from detrimental protein degradation. The 20S proteasome therefore has a low activity, at least toward folded protein substrates (17). Regulation of protein entry into the chamber is orchestrated by the regulatory particles, whose HbYX motif binds to pockets located between the α subunits thereby promoting the rearrangement of the N-terminal tails and opening of the gate (18). Four types of regulators were described (19): the 19S regulator (PA700); the 11S regulators PA28 $\alpha\beta$ (20, 21) and PA28 γ (22); and the PA200 regulator (Fig. 1A) (23). An additional particle, PI31 was shown to bind proteasomes (24); however, its effective contribution to proteasome function is still a matter of debate (25, 26). The 20S proteasome associated to the 19S regulatory particle forms the 26S proteasome. This complex degrades proteins tagged with polyubiquitin chains, which are attached to lysine residues. Ubiquitin-tagged proteins are recruited to the 19S regulatory particle, which recognizes the ubiquitin motif, then deubiquitinates, unfolds, and translocates protein substrates inside the catalytic chamber (17).

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⁴ The abbreviations used are: CTL, cytolytic T cell; ER, endoplasmic reticulum; PNGase, peptide N-glycanase.

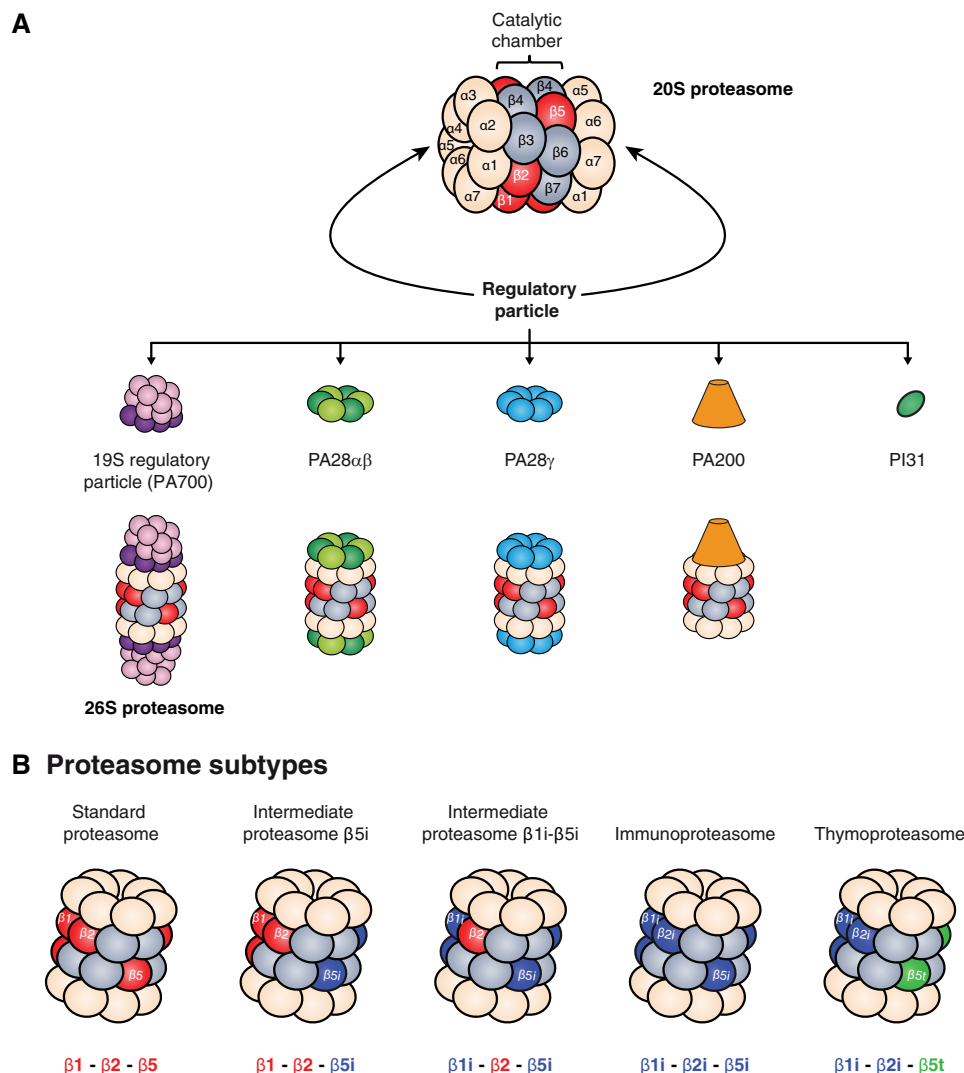


Figure 1. Structure and composition of proteasomes. A, structure of the proteasome and its regulators. The 20S proteasome is a cylindrical particle composed of four stacked heptameric rings ($\alpha_{1-7}-\beta_{1-7}-\beta_{1-7}-\alpha_{1-7}$) that delineate a chamber in which the proteins are degraded. The two outer rings are made of α subunits (beige), and the two inner rings are made of β subunits (red/bluish gray). Three types of β subunits are catalytically active (red). The α rings form the gates of the proteasome that control the entry of intracellular proteins inside the proteasome chamber. They can interact with different types of regulatory particles as follows: 19S regulatory particles (PA700), PA28 $\alpha\beta$, PA28 γ , PA200, and PI31. These regulatory particles can bind to one or both sides of the 20S particle or form hybrid proteasomes where the 20S core binds two different regulators. B, proteasome subtypes. Depicted are the five proteasome subtypes, which differ in their catalytic subunits. The standard proteasome contains the constitutive catalytic subunits $\beta 1$, $\beta 2$, and $\beta 5$, whereas the immunoproteasome contains the interferon- γ -inducible catalytic subunits $\beta 1i$, $\beta 2i$, and $\beta 5i$. The intermediate proteasomes express a mixed assortment of constitutive and inducible subunits; the intermediate proteasome $\beta 5i$ contains $\beta 1$, $\beta 2$, and $\beta 5i$ and the intermediate proteasome $\beta 1i-\beta 5i$ contains $\beta 1i$, $\beta 2$, and $\beta 5i$. Finally, the thymoproteasome contains catalytic subunit $\beta 5t$, which is homologous to $\beta 5$ and $\beta 5i$ and is specifically expressed in the cortical thymic epithelial cells along with $\beta 1i$ and $\beta 2i$.

The catalytic activity of the proteasome is centralized to three different β subunits of the proteasome: $\beta 1$, $\beta 2$, and $\beta 5$. As demonstrated by mutagenesis studies and analysis of the crystal structure of proteasome-bound inhibitor (14, 15, 27–29), peptide bond hydrolysis is initiated at the active site: the hydroxyl group found on the side chain of the N-terminal threonine of the catalytic subunit produces a nucleophilic attack on the carbonyl group of the peptide bond (Fig. 2A). This leads to the production of an acyl-enzyme intermediate in which the N-terminal peptide fragment remains attached to the catalytic threonine through an ester link. This acyl-enzyme intermediate is then rapidly hydrolyzed by water molecules present in the proteasome chamber, releasing the peptide fragment, which then exits the proteasome and is transferred into the cytosol (30). Three major proteasome catalytic activities were described: the

caspase-like activity, the chymotrypsin-like activity, and the trypsin-like activity, which cleave after acidic, hydrophobic, and basic amino acids, respectively. In the standard proteasome, the caspase-like activity is assigned to the $\beta 1$ subunit, the trypsin-like activity to $\beta 2$, and the chymotrypsin-like activity to the $\beta 5$ subunit. However, these assignments are not absolute, because amino acids surrounding the cleavage site might also influence the cleavage efficacy. More specifically, it is the nature of the amino acids that line the structural pockets around the active site, as well the size and the structure of these pockets that dictate the cleavage characteristics of each subunit (31).

Peptide splicing in the proteasome

Several years ago, Hanada *et al.* (7) made the unexpected observation that a CTL clone directed against a renal cell car-

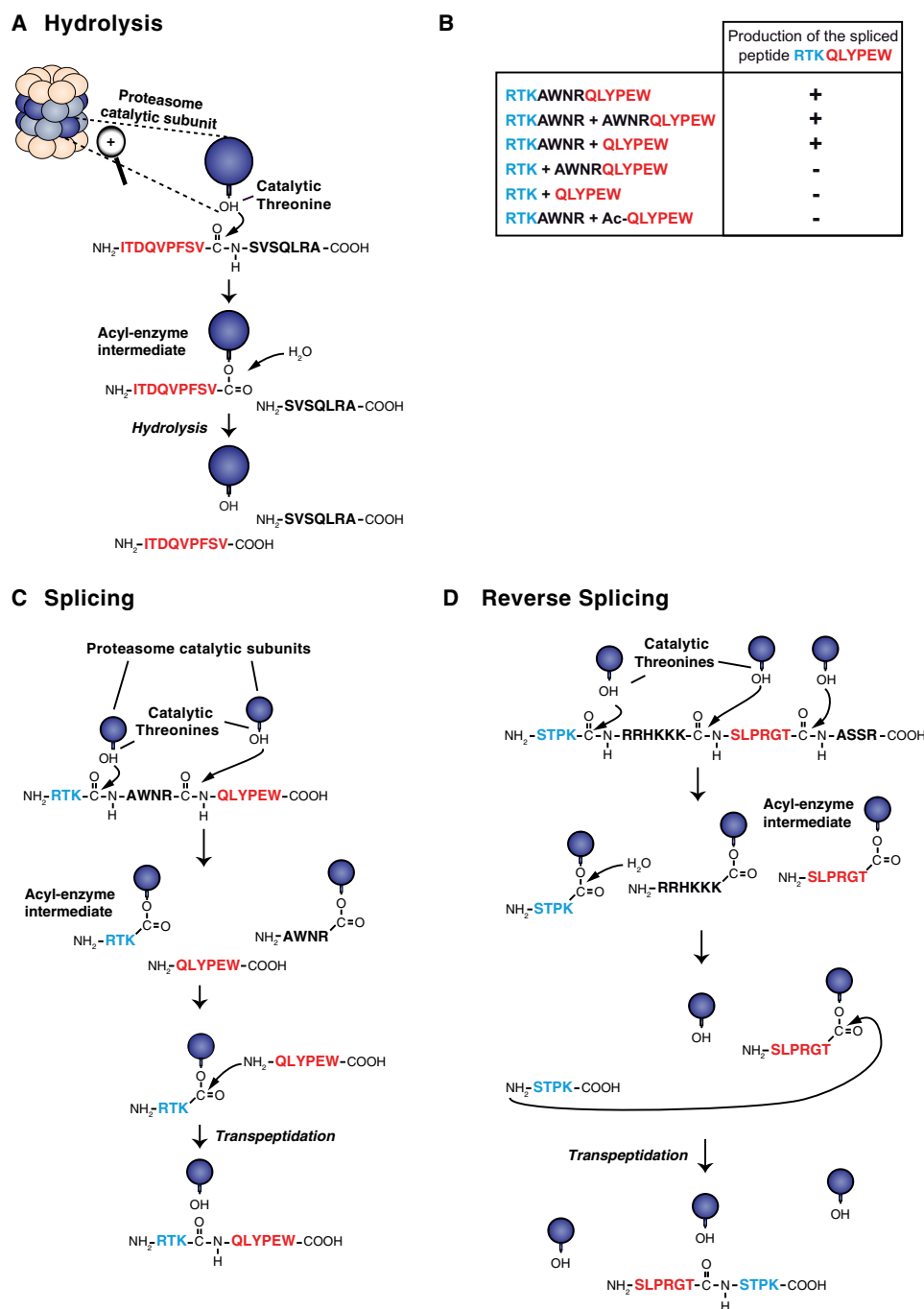


Figure 2. Proteasome catalytic activities. A, hydrolytic activity of the proteasome. The hydroxyl group of the N-terminal threonine of the catalytic subunits of the proteasome attacks the carbonyl group of the peptide bond. This leads to the production of an acyl-enzyme intermediate, in which a peptide fragment remains attached to the proteasome by an ester link. Then a water molecule hydrolyzes the ester link between the peptide and the threonine residue, thereby restoring the hydroxyl group of the catalytic threonine and producing the C-terminal end of the antigenic peptide. B, peptide splicing occurs by transpeptidation. Various synthetic peptides were combined in a pairwise manner and incubated with 20S proteasomes. The presence of the peptide RTK_QLYPEW in the digests was determined using either a specific CTL or by mass spectrometry. The presence (+) or absence (–) of the peptide in the digest is indicated. Ac-QLYPEW, N-α-acetylated peptide QLYPEW. C, peptide splicing in the proteasome. Here, the splicing of the antigenic peptide RTK_QLYPEW derived from the gp100 is shown. Following formation of the acyl-enzyme intermediate involving the fragment RTK, the free N-terminal amino group of peptide QLYPEW present in the proteasome chamber attacks the acyl-enzyme intermediate leading to the formation of the peptide RTK_QLYPEW composed of two peptide fragments originally distant in the protein. D, reverse splicing reaction in the proteasome. The splicing of the antigenic peptide SLPRGT_STPK derived from the SP110 is shown. Following formation of the acyl-enzyme intermediate involving the fragment SLPRGT, the free N-terminal amino group of peptide STPK attacks the acyl-enzyme intermediate leading to the formation of the peptide SLPRGT_STPK. Because in the protein SP110, the C-terminal reactant STPK is located upstream from the N-terminal reactant SLPRGT, the final peptide is composed of two peptide fragments originally distant in the protein and spliced together in reverse order to that in which they occur in the protein.

cinoma recognized a peptide composed of two fragments originally distant in the FGF-5 protein (Table 1). Production of this peptide required the removal of a 40-amino acid-long intervening sequence and the splicing of the peptides located at either

end. A second spliced peptide was discovered a few months later, when we isolated, from the blood of a melanoma patient, a CTL that recognized a peptide containing two non-contiguous fragments of the melanoma differentiation antigen

Table 1
Spliced peptides

Peptide source	Peptide sequence	MHC restriction	Peptide splicing partners and intervening sequence	SP ^a	IP ^a	Refs.
Spliced peptide						
FGF-5(172–176 and 217–220)	NTYAS_PRFK	HLA-A3	NTYASAIHRTEKTGREWYVALNKRKGAK RGCSRVKPKQHISTHFLPRFK	+	–	7, 10, 47
gp100(40–42 and 47–52)	RTK_QLYPEW	HLA-A32	RTKAWNRQLYPEW	+	+ / –	8, 47
gp100(47–52 and 40–42)	QLYPEW_RTK	HLA-A3	RTKAWNRQLYPEW	ND ^b	ND	13
gp100(195–202 and 191 or 192)	RSYVPLAH_R	HLA-A3	(R)RGSRSYVPLAH ^c	+	ND	12
Tyrosinase(368–373 and 336–340)	IYMDGT_ADFS	HLA-A24	ADFSFRNTLEGFASPLTGIADASQSSMHN ALHIYMDGT ^d	+	–	11, 47
SP110(296–301 and 286–289)	SLPRGT_STPK	HLA-A3	STPKRRRHKKKSLPRGT	+ / –	+	9, 47

^a SP indicates standard proteasome; IP indicates immunoproteasome.^b ND means not determined.^c Either Arg-191 or Arg-192 can be used to produce the final spliced peptide.^d The deamidated aspartates are underlined.

gp100 (Table 1) (8). The presence of this peptide in the peptidic eluates obtained from surface HLA molecules confirmed its natural presentation by melanoma cells. The use of proteasome inhibitors suggested that the proteasome was responsible for the splicing of these two fragments. This could be confirmed by incubating purified proteasomes with an unspliced peptide precursor (RTKAWNRQLYPEW); this led to the production of the spliced peptide (RTK_QLYPEW), which could be identified in the digest by mass spectrometry or with the CTL when the digests were pulsed onto antigen-presenting cells. By incubating pairs of peptides containing portions of the precursor peptide RTKAWNRQLYPEW with purified proteasomes (Fig. 2B), we demonstrated that peptide splicing involves a transpeptidation reaction based on the formation of an acyl-enzyme intermediate between fragment RTK and the proteasome. Instead of being hydrolyzed, the ester bond of the acyl-enzyme intermediate is subjected to a nucleophilic attack, driven by the free amino group of the peptide QLYPEW (Fig. 2C). This model was supported by the fact that *N*- α -acetylation of the QLYPEW peptide incubated with RTKAWNR and proteasomes completely prevented production of the spliced peptide (Fig. 2B). This reaction leads to the creation of a new peptide bond between the fragment RTK and QLYPEW thereby forming peptide RTKQLYPEW recognized by the CTL. Similarly, the first spliced peptide identified by Hanada *et al.* (7) was also shown to be produced by peptide splicing in the proteasome, despite the length (40 amino acids) of the intervening peptide sequence (10).

A few years later, our group identified a third spliced peptide, which corresponded to a minor histocompatibility antigen encompassing a polymorphism in the SP110 nuclear protein, and was recognized by a CTL isolated from a recipient of MHC-matched allogeneic hematopoietic cell transplantation (Table 1) (9). Interestingly, this peptide contained two non-contiguous fragments of the SP110 protein that were spliced together in the reverse order to that in which they occur in the parental protein. Again, the peptide splicing reaction was shown to take place in the proteasome by transpeptidation (Fig. 2D).

Because in the three peptides described above, the size of the intervening sequence varied from 4 to 40 amino acids, we have tested whether the length of the intervening sequence could affect the efficiency of peptide splicing (10). To do this, we transfected constructs in which the two peptides to be spliced were separated by intervening sequences of different lengths,

		Production of the spliced peptide NTYASPRFK (IFN γ produced by CTL C2 pg/ml)
FGF-5 wt	...NTYASAIH...THFLPRFK...	1946
FGF-5 Y174A	...NTAASAIH...THFLPRFK...	26
FGF-5 R218A	...NTYASAIH...THFLPAFK...	26
FGF-5 Y174A + FGF-5 R218A	...NTAASAIH...THFLPRFK... ...NTYASAIH...THFLPAFK...	67

Figure 3. Lack of physiological relevance of trans-splicing. Shown is an experiment designed to visualize the occurrence of splicing of fragments originating from two different proteins (*i.e.* trans-splicing). COS-7 cells were transfected with pairs of constructs mutated at critical residues so that the only way to produce the antigenic peptide was to splice fragments originating from two different proteins. The amount of IFN γ produced by the CTL after incubation with the transfected cells is shown. The results indicate that trans-splicing does not occur at a significant level in cells.

and we found that the shorter the intervening sequence, the higher the efficiency of peptide splicing. In addition, to test in cells whether the peptide splicing could occur between peptides located on distinct parental proteins, we transfected COS-7 cells with pairs of constructs that were mutated at critical residues so that the only way to produce the relevant antigenic peptide was to splice peptide fragments originating from two distinct proteins (Fig. 3) (10). We tested CTL recognition of the transfected cells and concluded that splicing of fragments originating from two different proteins (*i.e.* trans-splicing) hardly occurred (Fig. 3). Given that the very low level of trans-splicing observed in these experimental conditions was favored by the overexpression of the transfected constructs in COS-7 cells, we concluded that trans-splicing was unlikely to occur at all in physiological conditions. This might be linked in part to the fact that two distinct protein substrates can barely fit inside the same proteasome particle at the same time. Moreover, the likelihood of having two relevant protein substrates repetitively degraded at the same time inside one proteasome particle is extremely low.

A fourth example of spliced peptide was identified when searching for the target of a tumor-infiltrating lymphocyte isolated from a melanoma patient; our group demonstrated that reverse splicing of two peptide fragments originating from the melanoma differentiation protein tyrosinase produced this

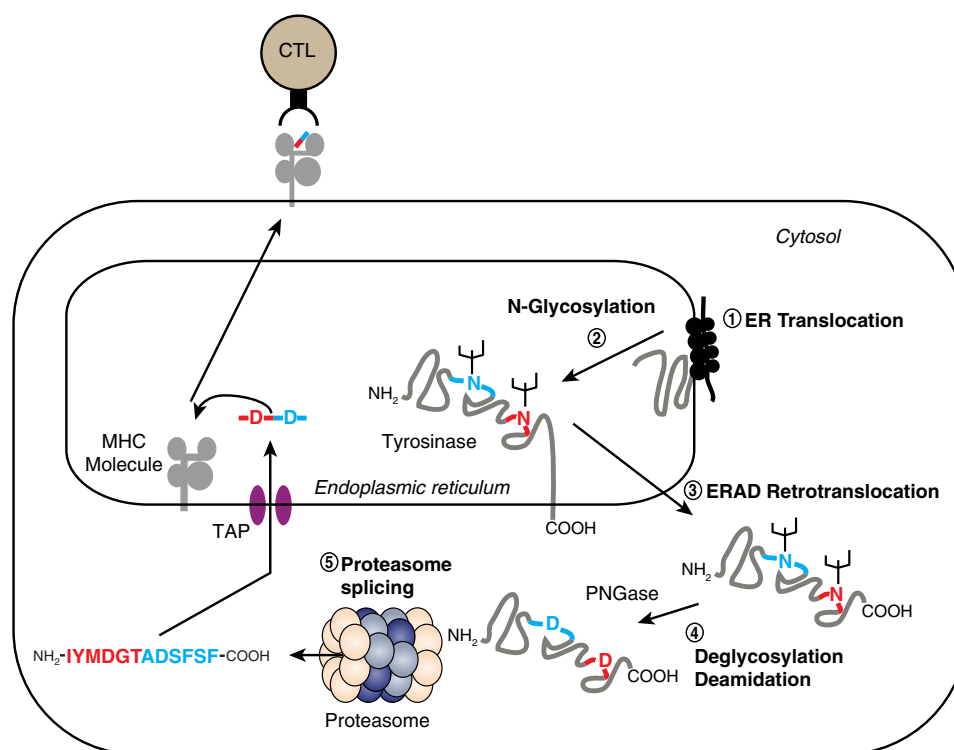


Figure 4. Production of the tyrosinase peptide IYMDGTADFSF. Upon entry into the ER (step 1), tyrosinase is glycosylated at specific asparagine residues (step 2). Some of the glycosylated tyrosinase protein is then retrotranslocated from the ER into the cytosol, likely due to misfolding (step 3). Upon deglycosylation by PNGase, asparagines are deamidated into aspartate residues (step 4). The aspartate-containing tyrosinase is then degraded by the proteasome (step 5), a process that leads to the reverse splicing of two fragments, each containing a post-translationally modified aspartate.

peptide (Table 1) (11). Surprisingly, the peptide identified also contained two additional post-translational modifications resulting in the conversion of two asparagine residues into aspartates by deamidation (Fig. 4). Deamidation of asparagine residues was previously suggested to occur following retrotranslocation of glycosylated tyrosinase into the cytosol (32); upon deglycosylation of glycosylated asparagines by peptide *N*-glycanase (PNGase), asparagines are deamidated into aspartates. The treatment of tumor cells with the PNGase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (32, 33) confirmed that PNGase was responsible for the conversion of the asparagines into aspartate residues in the new tyrosinase spliced peptide (11). Following deamidation, the aspartate-containing tyrosinase protein is degraded by the proteasome, a process that leads to the reverse splicing of two fragments, each containing a post-translationally modified aspartate (Fig. 4) (11).

More recently, a fifth spliced antigenic peptide was identified by our group, and was derived from the gp100 protein (12) (Table 1). In contrast to previous examples of spliced peptides, which were produced by the association of fragments containing 3–6 amino acids, this new peptide resulted from the association of an N-terminal 8-amino acid fragment with a single arginine residue at the C terminus. However, the analysis of proteasome digests obtained with peptide fragments of different lengths indicated that the transpeptidation could only occur when the fragment performing the nucleophilic attack on the acyl-enzyme intermediate, *i.e.* the C-terminal splice reactant, was at least 3 amino acids in length. Our results thus indicated that this spliced peptide was produced in two steps. A

transpeptidation first produces a spliced peptide elongated at the C terminus. This elongated peptide is then cleaved, likely also by the proteasome, to produce the final spliced antigenic peptide (12). This mechanism allows the production of spliced peptides encompassing a C-terminal fragment as short as 1 or 2 amino acids. It is highly likely that the N-terminal spliced reactant, *i.e.* the fragment involved in the formation of the acyl-enzyme intermediate, also has a minimal length even though this was not formally tested. However, N-terminal trimming of antigenic peptides is an efficient process that takes place in the ER and is orchestrated by ER-resident amino peptidase (3, 34). Therefore, spliced peptides involving short N-terminal fragments most likely exist and result from the production of N-terminally elongated spliced peptides that are further trimmed in the ER.

Identification of spliced peptides through computational approaches

In essence, the splicing of peptides by the proteasome appears as a by-product of the process of protein degradation by the proteasome. In principle, splicing could involve any peptide fragment released through proteolysis and should produce a large variety of spliced peptides, albeit in relatively low amounts. Yet, until recently, very few spliced peptides were identified, and the identification of spliced antigenic peptides was often limited by the availability of specific CTL directed against these peptides. Mass spectrometry approaches were limited by the fact that peptide databases used to match ions only contain linear peptide fragments. To circumvent this problem, Liepe *et al.* (35) developed Splicemet, an algorithm

that allows the identification of spliced peptides in peptide digests. This algorithm elaborates a dataset compiling the m/z values of any spliced and non-spliced peptide that could be theoretically generated by the proteasome from a given precursor peptide. This theoretical dataset is then compared with the m/z values of the peptide fragments obtained after *in vitro* digestion of the corresponding synthetic precursor with 20S proteasome. The presence of a specific peptide in the digest is then verified by tandem mass spectrometry. Using this approach, a new spliced peptide, QLYPEW_RTK, derived from gp100 was identified (Table 1) (13). Interestingly, this peptide is composed of fragments that are identical to those contained in the previously described spliced peptide RTK_QLYPEW, but these fragments are spliced in reverse order. After stimulation of CD8⁺ T cells with this peptide, Ebstein *et al.* (13) isolated an HLA-A3-restricted CTL, which recognized the peptide and was able to kill gp100-expressing tumor cells. Because they observed that the peptide QLYPEW_RTK could be produced *in vitro* when incubating peptide fragment RTK and QLYPEW together with the proteasome, they suggested that the peptide could be produced *in vitro* through a proteasome-catalyzed condensation reaction; indeed the small and “final” size of those two fragments (RTK and QLYPEW) precludes the production of an acyl-enzyme intermediate that is required for the transpeptidation reaction. So far, however, the experimental evidence relative to the mechanism of condensation is limited to results obtained with *in vitro* digests or with cells expressing appropriately truncated constructs, but not with cells expressing full-length proteins (13). Moreover, the prominent role of transpeptidation over condensation was confirmed by mass spectrometry analysis of peptide digests performed in the presence of isotopically-labeled water (36).

In recent years, great progress in peptidomics has allowed the detailed characterization of peptides presented by the MHC class I molecules at the cell surface, based on the mass spectrometry analysis of peptides eluted from MHC molecules. Again, a major limitation in the identification of spliced peptides by peptidomics is the fact that databases used for matching only contain linear protein fragments. Recently, Liepe *et al.* (37) addressed this issue by creating a custom peptide database, which includes any possible short peptides created by the splicing of non-contiguous peptide fragments from the proteins expressed in the cell lines studied. To restrict the size of the database, only peptides produced by the cis-splicing of fragments separated by 25 amino acids or less were taken into account. When analyzing the HLA-I immunopeptidome obtained from different human cell lines, they showed that spliced peptides represented about 25% of the total amount of peptides found at the cell surface, suggesting that peptide splicing significantly contributes to the diversity of the peptide repertoire. Surprisingly, analysis of the peptides contained in the eluates showed that some proteins were only represented by spliced peptides and not by linear epitopes. This would suggest that these proteins generally lack linear peptides with adequate HLA-binding motifs or produce generally smaller peptides, so that splicing is the only way to produce peptides of adequate length and motif for MHC class I binding. However, it could also be that linear peptides from these proteins are displayed at

the cell surface in amounts that are below the detection threshold of mass spectrometry.

Additionally, when Liepe *et al.* (37), using NetMHC, analyzed the binding affinity of spliced peptides for the HLA-A and -B molecules from which they were eluted, they observed that their IC₅₀ was quite high when compared with non-spliced peptides. The authors suggested that this low predicted binding affinity might reflect intrinsic differences in the HLA-binding motifs of spliced *versus* non-spliced peptides, which are not captured by existing algorithms because these have been exclusively trained on non-spliced epitopes. In the future, one should try to compare experimentally measured binding affinities of a large series of spliced and non-spliced peptides to solve this issue. Moreover, it will be interesting to see whether other groups can confirm the presence of such a large population of spliced peptides at the cell surface, especially on tumor cells.

Peptide splicing by isopeptide linkage

Studying *in vitro* peptide digests, Berkers *et al.* (38) showed that the ϵ -amino group of lysine residues could also perform a nucleophilic attack on the acyl-enzyme intermediate, leading to the formation of a new type of spliced peptide containing an isopeptide bond (hereafter called spliced isopeptide). Surprisingly, their results show that splicing by ϵ -amines was only 10-fold less efficient than splicing by α -amines. Spliced isopeptides were shown to bind HLA molecules with high affinity, as long as the isopeptide bond is located in the middle or at the N terminus of the peptide. Strikingly, the presence of an isopeptide bond was shown to protect the spliced isopeptide from proteasome degradation. This increased stability of spliced isopeptides might have actually facilitated their identification in the first place but somewhat biased the evaluation of the splicing efficiency by ϵ -amines compared with α -amines; in their experiments, Berkers *et al.* (38) performed proteasome digestions over 16–24-h periods, which might have favored the secondary degradation of spliced peptides over spliced isopeptides, thereby artificially increasing the production rate of these spliced isopeptides compared with the canonical spliced peptides. Moreover, although spliced isopeptides were shown to activate patient-derived CD8⁺ T cells *in vitro*, the presence of spliced isopeptides at the cell surface has not yet been demonstrated, questioning whether this phenomenon really occurs in physiological conditions.

Deciphering the rules of peptide splicing by the proteasome

Predicting algorithms have for long been a focus of research, with the aim to facilitate the identification of potential targets in the immunotherapeutic context. Proteasome cleavage prediction algorithms, which are generally trained either on *in vitro* proteasome digestion datasets or on described MHC class I ligands (whose C terminus reflects the proteasome cleavage site), have been proposed (39). So far, however, their reliability has not been firmly established. Recently, several groups have tried to highlight various parameters that drive peptide splicing by the proteasome. A first approach, developed by Mishto *et al.* (36), is based on the *in vitro* digestion of extended peptide precursors and the systematic identification and quantification of

the spliced and the non-spliced peptide fragments released in the course of the digestion. This led to the notion that one of the rate-limiting factors that drive peptide splicing by the proteasome is the quantity of the spliced reactants involved. In particular, the splicing reaction is limited by the amount of the less abundant reactant, regardless whether this fragment ends up localizing at the N- or the C-terminal side of the final antigenic peptide. Unexpectedly, however, Mishto *et al.* (36) did not observe any correlation between the strength of proteasome cleavage at a given position and the frequency at which the corresponding amino acid is used to compose either the N- or the C-terminal residues of the spliced reactants, *i.e.* P1' and P1, respectively (Fig. 5). On the contrary, P1 and P1' residues were shown to often derive from minor cleavage sites, suggesting that splicing is not a random process but rather depends on the sequence of the spliced reactants. This led the authors to conclude that additional constraints determine the splicing efficiency. In particular, they postulated the existence of an additional pocket, distinct from the primed substrate-binding site, that could accommodate the C-terminal splice reactant and facilitate the peptide-splicing process. It follows that the ability of the C-terminal splice reactant to bind to this pocket (which is linked to its amino acid sequence) should determine its potential involvement in a peptide splicing reaction.

The notion that splicing does not follow random rules was further supported by recent work by Berkers *et al.* (40), who used short peptide libraries to identify the amino acid sequences that could promote the splicing reaction by the proteasome. Their results indicated that the splicing reaction was driven by the sequence of the N-terminal reactant and by the concentration rather than the sole sequence of the C-terminal ligation partner. Noteworthy, it remains difficult to determine whether the nature of the N-terminal splice reactant is really affecting the splicing reaction itself, or whether it simply reflects the affinity of the N-terminal reactant for the non-primed binding pockets of a particular catalytic subunit and thereby the ability of the acyl-enzyme intermediate to form. In that respect, sequence requirement for splicing is likely to differ between the three catalytic subunits of the proteasome as much as they do for cleavage. This makes it very difficult to compare the splicing of peptides with one or two amino acid substitutions, as these may target the peptide to another catalytic subunit, and the C-terminal reactant itself might not access as efficiently to the primed binding site of each catalytic subunit. Moreover, when Liepe *et al.* (37) compared spliced peptides eluted from distinct cell lines, they observed significant differences in the nature of the amino acids found at key positions (P₁, P₁' (Fig. 5), N terminus, and C terminus). They suggested that the nature of the amino acid found at these positions is also influenced by the HLA molecule to which these peptides bind (37). These biases clearly need to be taken into account when analyzing the rules of peptide splicing using the sequence of spliced peptides bound to HLA class I molecules.

Proteasome subtypes and peptide splicing

Immune cells and cells treated with inflammatory cytokines, such as IFN γ , express alternative proteasome catalytic subunits

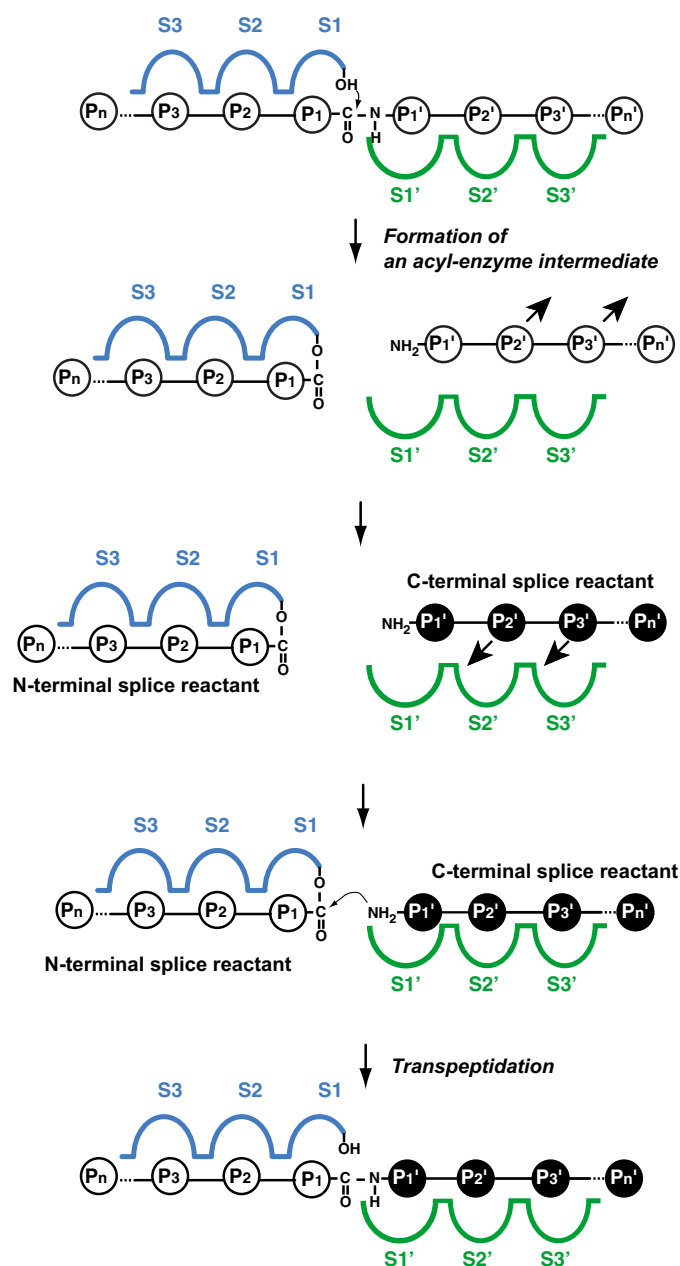


Figure 5. Schematic representation of the splicing reaction occurring at the catalytic site of the proteasome. The peptide to be cleaved is represented by a necklace with pearls indicating individual amino acids. The non-primed binding site is indicated in blue and the primed binding site is in green. Each binding site is subdivided in different pockets. Three pockets are labeled S1 to S3 for the non-primed site and S1' to S3' for the primed binding site. Amino acids binding to the non-primed binding site are labeled P₁ to P_n, and those binding to the primed binding site are labeled P₁' to P_n'. The peptide splicing reaction starts with the formation of an acyl-enzyme intermediate with the peptide located at the non-primed binding site. In the course of the peptide splicing reaction, the C-terminal splice reactant (black) comes into the vicinity of the acyl-enzyme intermediate, most likely by binding to the primed binding site. An alternative pocket that could fit the C-terminal reactant has been postulated but is not represented here. The amine group of the C-terminal splice reactant then produces a nucleophilic attack on the ester bond of the acyl-enzyme intermediate to create the spliced peptide, which then exits the catalytic site.

LMP2(β 1i), MECL-1(β 2i), and LMP7(β 5i), which are incorporated into proteasomes instead of β 1, β 2, and β 5, forming an alternative proteasome subtype, called the immunoproteasome (Fig. 1B). Because standard proteasomes and immunoprotea-

somes are composed of distinct catalytic subunits, their cleavage specificities are different, as observed by their activity on fluorogenic peptides: the immunoproteasome displays a much lower caspase-like activity, whereas its trypsin-like and chymotrypsin-like activities are increased (41, 42). This can be explained by subtle conformational changes in the substrate-binding pockets of catalytic subunits $\beta 1i$ and $\beta 5i$ (31). The S1 substrate-binding pocket (which interacts with the amino acid residue forming the C-terminal end of the cleaved peptide) of $\beta 1i$ is smaller, more hydrophobic, and lacks a positively charged residue (Arg-45) when compared with that of $\beta 1$, thereby accounting for the reduced caspase-like activity of $\beta 1i$ and its higher cleavage activity after small hydrophobic and branched residues (31). Likewise, $\beta 5i$ harbors a more spacious S1 pocket than $\beta 5$, favoring the cleavage after large non-polar residues like tyrosine, tryptophan, or phenylalanine (31). Because of its increased propensity to cleave after basic and hydrophobic amino acids, the immunoproteasome has been predicted to produce peptides that are more efficiently presented by MHC class I, which often call for basic and hydrophobic C termini for efficient binding. This idea was corroborated by the study of mouse knock-outs for all three immunosubunits in which presentation of a number of MHC class I epitopes was decreased (43). However, this notion was challenged by the fact that some antigenic peptides were shown to be more efficiently produced by the standard proteasome than by the immunoproteasome (41, 44–46). The current understanding is that proteasome subtypes produce distinct repertoires of peptides that are partially overlapping. The inefficient processing by one proteasome subtype generally results either from the incapacity of that proteasome to produce the C-terminal cleavage required to release the antigenic peptide or from the fact that it produces a prominent destructive cleavage inside the peptide sequence (45). Because the splicing is directly linked to the proteolytic activity of the proteasome, standard proteasome and immunoproteasome are both expected to produce spliced peptides. This was clearly demonstrated when we compared the processing of four spliced antigenic peptides by the standard proteasome and the immunoproteasome (47). We showed that peptides derived from FGF-5, gp100, and tyrosinase were all better processed by the standard proteasome, whereas the peptide derived from the minor histocompatibility SP110 was better produced by the immunoproteasome (Table 1). Here also, differences in the production of the spliced peptides resulted from a balance between production and destruction of the peptidic partners involved in the splicing reaction.

A few years ago, our group identified two additional types of proteasome (Fig. 1B), which contain only one ($\beta 5i$) or two ($\beta 1i$ and $\beta 5i$) of the three catalytic subunits of the immunoproteasome (41). These intermediate proteasomes are expressed in a large variety of cell types; they represent 10–20% of the total proteasomes found in tumors and 30–50% of those found in liver, kidney, small bowel, colon, and dendritic cells (41). Studying the catalytic activity of intermediate proteasomes, we showed that intermediate proteasome $\beta 1\beta 2\beta 5i$ (hereafter called intermediate proteasome $\beta 5i$) displayed a chymotrypsin-like activity that was intermediate between that of the standard and the immunoproteasome, whereas that of intermediate pro-

teasome $\beta 1i\beta 5i$ was similar to the immunoproteasome (41, 42). The trypsin-like activities of both proteasomes $\beta 5i$ and $\beta 1i\beta 5i$ are intermediate between that of the standard and the immunoproteasome. As expected, the caspase-like activity, which is linked to the presence of the $\beta 1$ subunit, is exhibited by intermediate proteasome $\beta 5i$ but not by proteasome $\beta 1i\beta 5i$. Because of their particular cleavage properties, intermediate proteasomes produce a unique repertoire of peptides (41, 46). Additionally, a fifth type of proteasome was found in the thymus (48, 49). This proteasome, called thymoproteasome, contains catalytic subunit $\beta 5t$, in place of $\beta 5$ – $\beta 5i$, and is expressed exclusively in thymic cortical epithelial cells (Fig. 1B). Subunit $\beta 5t$ is associated with $\beta 1i$ and $\beta 2i$ in the thymoproteasome, which displays a lower chymotrypsin-like activity and seems to produce a distinct peptide repertoire as compared with the immunoproteasome (50). Whether the intermediate proteasomes and the thymoproteasome are able to produce spliced peptides has not yet been demonstrated, but the inherent way by which spliced peptides are produced suggests that all proteasome types can produce spliced peptides, as long as they can form the appropriate acyl-enzyme intermediate and C-terminal reactant(s).

Moreover, this also implies that spliced peptides are not restricted to tumor cells but are also produced by healthy cells as long as they contain the appropriate type of proteasome; healthy melanocytes indeed express the spliced peptide derived from tyrosinase and can be recognized by the anti-tyrosinase CTL (11). Spliced peptides therefore do not form a specific category of tumor-associated antigens, but rather they behave like any other “classical” antigen against which central tolerance is potentially established in the thymus. Notably, proteasomes isolated from yeast can also produce spliced peptides (36).

Interestingly, peptide splicing was also involved in the production of MHC class II-restricted antigens recognized by diabetogenic CD4⁺ T cell clones in non-obese diabetic mice (51, 52). Interestingly, the MHC class II spliced peptides identified are produced by the splicing of fragments originating from two different proteins (trans-splicing), which are abundant in the secretory granules of β cells. Although the enzyme responsible for the splicing reaction was not yet identified, it likely corresponds to a protease found in the secretory compartment. In contrast to proteasome-catalyzed peptide splicing, splicing involving abundant secretory proteins may not require enzyme-procured compartmentalization, and likely explains why production of antigenic peptides by trans-splicing is physiologically possible in this context.

Conclusions

CTLs are major sentinels devised to rapidly recognize and destroy cells expressing mutant, infectious or tumoral proteins. Their ability to recognize as few as one to five peptide–MHC complexes at the cell surface provides them with a high sensitivity that enables the detection of cells displaying only subtle changes in their cellular protein content (53). Peptide splicing is a low efficiency process; it was estimated that *in vitro*, 1–2% of the total fragments produced by proteasome-mediated degradation represent spliced peptides (36). Yet, a number of those

peptides were found to elicit meaningful CTL responses in cancer patients (7–9, 12), leading to tumor regression in some patients (11). Because the splicing activity of the proteasome is directly linked to its catalytic activity, its efficacy likely follows similar rules, based on the way peptide partners interact with the primed and unprimed pockets at the catalytic site, including their affinity for the pockets, their off-rate, and the orientation of the attacking peptide in the substrate-binding channel, which might increase its nucleophilicity (54).

Despite the low efficiency of peptide splicing, Liepe *et al.* (37) reported that the amount of spliced peptides found at the cell surface represented about 25% of the total amount of peptides displayed. If confirmed, this striking observation would raise questions on how such a low-efficiency process might produce so many MHC-I-presented peptides. Is it because the spliced peptides are more efficiently transported through the MHC class I pathway? Do these peptides have increased binding affinities for MHC class I molecules, thereby endowing them with an increased competitiveness for HLA binding compared with other peptides? Do proteasome regulators play a role in the efficiency of the splicing process (for example, by differentially affecting the sequestration of potential C-terminal splice reactants inside the catalytic chamber)? Additional studies will be required to better understand the ins and outs of the regulation of peptide splicing by the proteasome.

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Peptide splicing by the proteasome

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