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REVIEW

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Novel biochemistry: post-translational protein splicing and other lessons from the school of antigen processing

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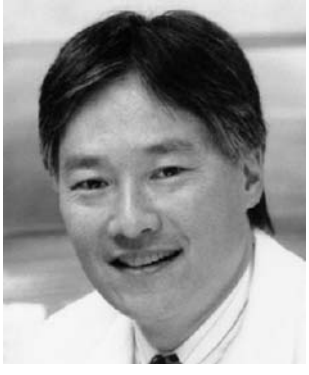
Abstract In the past 15 years, the molecular identification of antigens that can mediate the killing of tumor cells by T cells has been vigorously pursued. Molecular identification of tumor-associated antigens not only provided the means to activate or monitor anti-tumor immunity, but also gave insights into new and unexpected biochemical processes that are taking place within cells. Post-translational splicing, a phenomenon previously identified only in lower organisms or plants, has recently been added to the list of atypical processes generating proteins in humans. The proteasome, whose main function is to degrade intracellular proteins, appears to catalyze this splicing reaction. The discovery of post-translational splicing has immediate and important implications for the complexity of the major histocompatibility complex (MHC) class I peptide repertoire and for the immune recognition of self- and foreign peptides.

Keywords Protein splicing · Tumor antigens · Antigen presentation



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Introduction

Over the course of evolution, acquisition of an immune system that can survey intracellular and extracellular antigens enabled vertebrates to survive the threat of viral or bacterial infections. Immune surveillance of intracellular antigens, most likely to detect viral proteins with foreign amino acid sequences and eliminate infected cells, can also detect and destroy cancer cells expressing mutated or aberrantly expressed proteins [1]. Data from animal studies [2, 3] and clinical trials [4–6] support the contention that augmenting this T-cell response to tumors could be valuable as a treatment for patients with advanced cancer. However, in most cases, anti-tumor immunity is not robust enough to eradicate cancer, and some sort of manipulation is necessary to induce stronger anti-tumor immunity. This need to enhance existing anti-tumor T-cell responses led to efforts to define tumor antigens and epitopes recognized by tumor-reactive T cells, and this pursuit has uncovered some surprising insights into human biology. The majority of defined anti-tumor T-cell responses involve the processing of intracellular proteins, presented to $CD8^+$ T cells in the context of the class I major histocompatibility complex (MHC). This is in contrast to recognition of extracellular

antigens, engulfed from the environment by antigen-presenting cells and presented to $CD4^+$ T-cells in the context of the class II MHC. Such class II MHC restricted tumor recognition does occur and probably plays important roles in the regulation of anti-tumor responses through the activation of dendritic cells by $CD4^+$ $CD25^-$ helper T cells [7, 8] or through the immunosuppression by $CD4^+$ $CD25^+$ regulatory T cells [9, 10]. However, the main anti-tumor effector mechanism in tumor rejection seems to involve CD8 responses, and a thorough understanding of how tumor-associated antigens are processed and presented in the context of the class I MHC is critical in tumor immunology.

Class I MHC antigen-processing system

The class I MHC is a family of pleomorphic type I membrane glycoproteins that belongs to the immunoglobulin superfamily and is expressed on nearly all the cells in the body. These molecules have the role of presenting peptides that are derived from intracellular proteins to $CD8^+$ T cells (Fig. 1). Intracellular proteins are degraded by proteases such as proteasome, tripeptidyl peptidase II, and other aminopeptidases in the cytoplasm [11]. Resultant short peptides (usually less than 12 amino acids) are transferred into the endoplasmic reticulum (ER) by the transporter for antigen processing in an ATP-dependent manner [12]. At this point, most of the peptides are too long to bind to class I MHC peptides, and they need to be further cleaved by enzymes such as ER aminopeptidase [13–15]. Then, peptides (usually 8–11 amino acids, typically 9 amino acids) bind in a groove between two α helices on the membrane-distal surface of a class I MHC protein [16] and are displayed on the cell surface. MHC molecules are promiscuous, and they bind many different peptides with certain amino acid preferences at key positions (anchor residues) which constitute binding motifs [17]. Different MHC alleles have different motifs and bind different sets of peptides. The number of class I MHC molecules per cell is estimated at 50,000–100,000 [18] and because as many as 2×10^6 peptides are estimated to be generated every second [19], only a small minority of peptide epitopes can be presented on a class I MHC at any time.

T-cell receptor-mediated antigen detection

Unlike antibodies, which bind with the intact folded antigen, T cells recognize only the specific, minimal determinant epitope processed from the antigen and displayed on the correct "restricting" MHC molecule [20] (Fig. 1). T cells that can recognize their cognate peptide/MHC complex only at high antigen density are termed low-avidity T cells, whereas those that recognize antigens at low densities are termed high-avidity T cells. This detection can

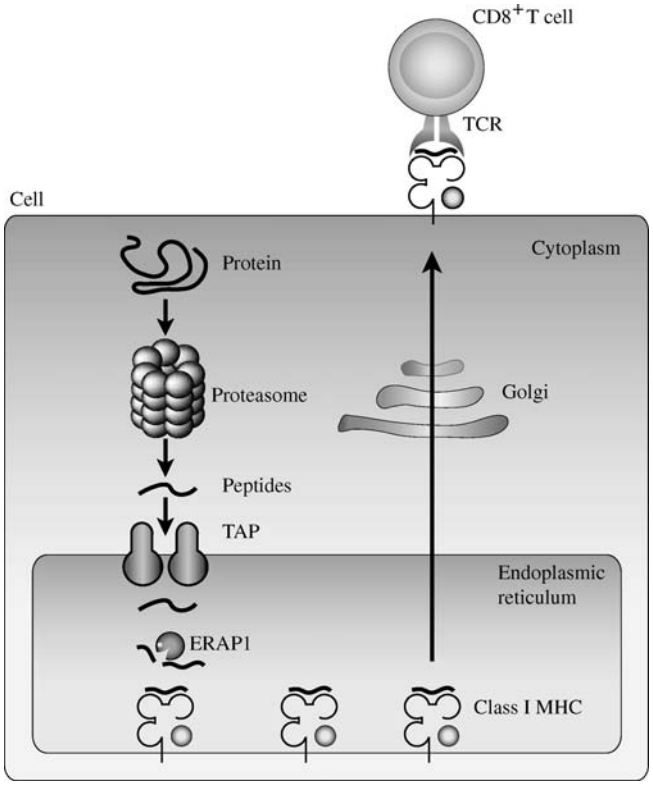


Fig. 1 Class I major histocompatibility complex (*class I MHC*) restricted antigen-presenting system. Cytoplasmic proteins are degraded by cytoplasmic peptidases such as the proteasome and tripeptidyl peptidase II. Resultant short peptides are transferred to the endoplasmic reticulum (ER) by a transporter for antigen processing (*TAP*) in an ATP-dependent manner. Peptides that are too long to be presented by class I MHC molecules are trimmed by ER aminopeptidases such as ERAP1. Peptides bound to class I MHC are presented on the cell surface and surveyed by $CD8^+$ T-cells

be exquisitely sensitive, with T cells recognizing antigen-presenting cells incubated in sub-nanomolar amounts of the correct minimal determinant peptide. However, it is not clear how many peptide/MHC complexes are necessary to trigger $CD8^+$ T cells. This can be influenced by such factors as T-cell receptor (TCR)–MHC affinity, peptide–MHC affinity, and the peptide–TCR affinity. Older studies showed that 100–400 complexes per antigen-presenting cell were necessary [21–23], but more recent work shows that the activation of a T cell can be initiated by three to five peptide–MHC complexes per antigen-presenting cell [24, 25]. In the most extreme example, it was reported that an average of three peptide–MHC complexes per target cell could elicit a half-maximal cytolytic T-cell response, and the response of some T-cells could be elicited by a target cell that bears a single peptide–MHC complex [26]. When tumor immunologists discovered means for isolating and cloning tumor-reactive T cells, they then proceeded to probe these tumors for the protein antigens mediating recognition, using the T cell and TCR as a sensitive peptide-detection system.

Tumor-associated antigens

Over the past decade, numerous tumor-associated antigens that are recognized by tumor-specific cytotoxic T lymphocytes (CTLs) have been identified [27]. The basic methodology uses a tumor-reactive T cell to screen a tumor cDNA library expressed in COS7 or 293 cells also transfected with the appropriate restricting MHC molecule. Reactivity resulting from combining the T cell with the target cells transfected with the correct cDNA is evidenced by IFN-γ release and is detected by ELISA. Tumor antigens recognized by T cells can be categorized into four main groups:

1. Cancer/testis antigens including MAGE-1 [28] and NY-ESO-1 [29]. These antigens are expressed in tumors, testis, and placenta but not other normal tissues.
2. Lineage-specific differentiation antigens including MART-1/Melan-A [30, 31], gp100 [32, 33], and PSA [34].
3. Proteins overexpressed by tumor compared to normal tissues such as fibroblast growth factor-5 (FGF-5) [35] and PRAME [36].
4. Tumor-specific mutated antigens such as β-catenin [37] and CDK-4 [38].

Over 50 antigens responsible for the recognition of tumor cells by T cells have been identified, most expressed by melanoma, which has a propensity for generating tumor-reactive T cells [27]. The next step in the clinical development of these immunogenic tumor-associated proteins is to identify means of enhancing the reactivity against these antigens in patients. Active immunization by vaccination [39, 40] and passive immunization by T-cell transfer [41] have both been pursued. Passive transfer of antigen-specific, activated T cells expanded in vitro has demonstrated that tumor-reactive T cells can cause major tumor regression. Vaccination is appealing in that it avoids the complexity and logistical obstacles of T-cell transfer. Unfortunately, it rarely causes tumor regression by itself, and the optimal means of vaccinating against these antigens is not established [42]. Thus far, the most consistent method of augmenting the number of CTL precursors in patients with cancer has been by immunizing repeatedly with a minimal determinant peptide in incomplete Freund's adjuvant. In addition, conservative substitutions in the native sequence of these minimal determinant peptides, which increase their affinity for their presenting MHC molecule while not affecting the TCR-contact residues, can dramatically augment their immunogenicity [43]. Therefore, the best current approach to immunization against cancer requires that the minimal determinant peptide be identified within the tumor antigens recognized by T-cells. The path from antigen identification to discovery of the minimal determinant peptides presented on specific MHC molecules has uncovered several novel biochemical processes previously unsuspected in the generation of proteins from the human genome. The exquisite sensitivity of the TCR as an instrument for detecting specific peptide sequences is primarily responsible for these new discoveries. Although many of the precise mechanisms creating these epitopes are not fully elucidated, and the functional significance of many of these findings is also unknown, they already have immediate implications within immunology.

Discovery of post-translational splicing in FGF-5

A renal cell carcinoma (RCC)-specific CTL clone (C2) was established from a metastatic lung lesion in a renal cell carcinoma patient that had shown spontaneous regression, a rare phenomenon thought to be immunologically mediated [35]. C2 recognized six of ten renal carcinoma cell lines expressing HLA-A3 and also some breast, bladder, and prostate cancer cell lines. By expression-based cDNA library screening, the antigen was identified as FGF-5 and it was also established that the recognition of cell lines by C2 was closely correlated with their expression of FGF-5 by quantitative reverse transcription-polymerase chain reaction (RT-PCR) [35]. These observations indicated two clinically important points: (1) the epitope is a non-mutated epitope that can be used for the vaccination of multiple patients and (2) utility of an FGF-5 vaccine may not be limited to RCC. In order to identify the minimal determinant epitope presented by HLA-A3, we prepared truncated versions of the *FGF-5* gene, asking whether these constructs retained the ability to confer recognition by the CTL when transfected into an HLA-A3$^+$ cell line. This is usually a routine process, but in the case of FGF-5, it was not [44]. 3′-truncations of the full-length *FGF-5* gene (Fig. 2a, G1) that encodes 269 amino acids (aa) showed that the fragment encoding $aa_{1–220}$ (Fig. 2a, G2) was recognized but the fragment encoding $aa_{1–212}$ (Fig. 2a, G3) was not. 5′-truncations showed that $aa_{161–220}$ (Fig. 2a, G4) included the epitope but $aa_{173–220}$ (Fig. 2a, G5) did not. These results indicated that the minimum fragment required to generate the epitope spanned at least aa_{172} and aa_{213} and perhaps as much as $aa_{161–220}$. However, a 42-aa peptide far exceeded any known class I MHC binding peptide. One hypothesis considered was that the epitope resided at one end of this fragment, and the remainder of this peptide was necessary for it to serve as substrate in an enzyme-mediated, post-translational modification. Synthesizing and testing all native 9-, 10-, and 11-aa peptides encoded within the 60-aa fragment ($aa_{161–220}$) resulted in no CTL triggering, supporting the possibility of post-translational modification. The working hypothesis suggested that some of the internal peptide sequence might not be necessary for peptide modification, so internally deleted mini-gene constructs were made and tested. One of these constructs was recognized by C2 (Fig. 2b, G6). Starting with this construct, we prepared stepwise single-codon truncation mutants and further identified a minimal sequence that was necessary for CTL recognition. These showed that a gene fragment that encoded $aa_{172–176}$ and $aa_{199–220}$ (Fig. 2b, G12) was as well recognized as full-length FGF-5. Next, within this truncated, internally deleted peptide, alanine (or glycine for alanine) substitutions at every amino acid position were

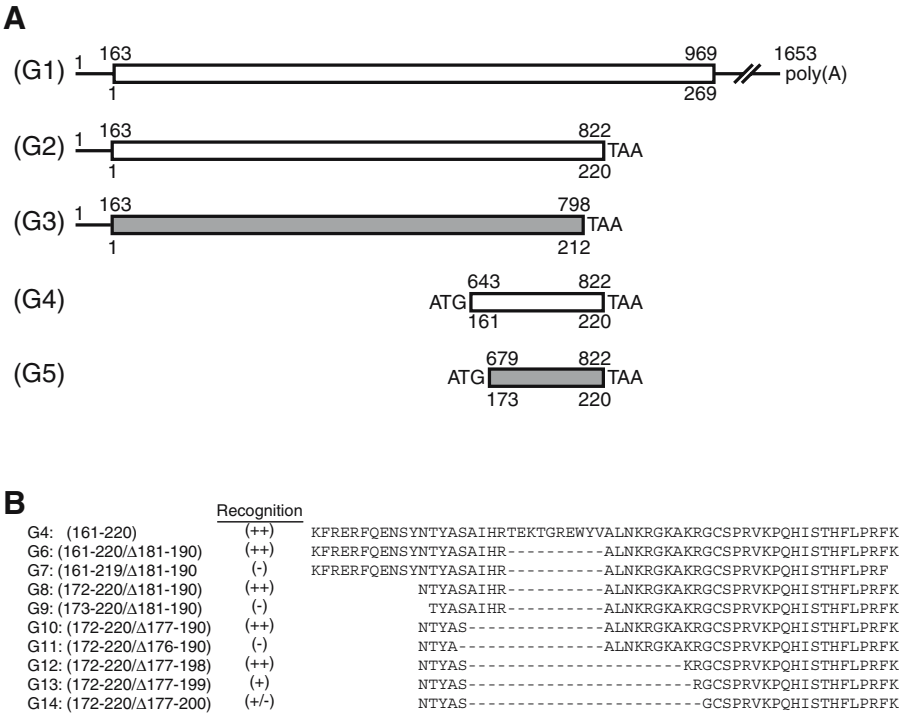


Fig. 2 (Adapted from [44]). **a** Truncation analysis of the *FGF-5* gene. A series of truncation mutants were prepared by polymerase chain reaction (PCR) from construct G1. Each PCR product was cloned into the eukaryotic expression vector and transfected into COS cells expressing HLA-A3. Recognition by C2 was assessed by IFN-γ secretion. Nucleic acid positions are indicated *above the start and finish of each construct* and the corresponding amino acid positions from FGF-5 *below*. Constructs shown as *open boxes* were recognized and those shown as *filled boxes* were not. **b** Analysis of internal deletion mutants. For simplicity, predicted amino acid sequences instead of DNA sequences are shown. *Numbers in parentheses* indicate the position of the first and the last amino acids. *Numbers after* Δ indicate internally deleted amino acid residues

introduced by site-directed mutagenesis to find out which amino acids were crucial for the recognition by C2. The results showed that, within the 27 amino acids, only the 1st, 3rd, 5th, and the last four amino acids were crucial. This observation indicated that, if there were a contiguous epitope at one end of the fragment and it was a 9-mer, nearly half of the amino acids in the epitope were interchangeable with alanine, which was a very unusual observation. The HLA-A3 peptide-binding motifs favor tyrosine at position 3 and phenylalanine or lysine at positions 9 or 10. Analysis of the critical amino acids in FGF-5 identified by alanine substitution showed a tyrosine at aa_{174} and lysine at aa_{220}. In fact, fusing the two discontinuous peptide segments that included all the crucial amino acids formed a neopeptide that conformed well to the HLA-A3 binding motif. This peptide, consisting of NTYAS from the N-terminus and PRFK from the C-terminus was synthesized and tested, and it strongly stimulated C2. Several avenues of investigation indicated that the fusion event was post-translational. First and foremost, a synthetic 49-aa peptide from FGF-5 that included the critical amino acid residues was successfully taken up, processed, and presented to C2. This was accomplished by metabolically active B-cell antigen-presenting lines, but not fixed B-cell lines (whereas the FGF-5 post-fusion 9-mer could be successfully presented by fixed antigen-presenting cells). Therefore, the processing event could only be mediated by a viable cell and was carried out on a peptidic precursor. It was also considered unlikely that RNA splicing was involved in that multiple mini-genes with differing internal deletions were previously made and could confer CTL recognition to target cells on transfection. The prediction was that these differing deletions should corrupt splice-donor and acceptor motifs and not all would result in successful splicing to yield the same 9-mer fusion peptide. To further rule out RNA splicing and translational mechanisms such as ribosome skipping [45], termination codons were introduced in three separate positions within the hypothetical intronic region, and all three stop codons interrupted epitope creation and CTL recognition. Finally, it was shown by HPLC that the epitope presented on the surface of a recognized tumor line and extracted by acid elution co-migrated with the candidate 9-mer epitope, not the 49-mer precursor. Only one mechanism can explain all these observations and that was that the epitope was generated by post-translational splicing. FGF-5 splicing seems to be a ubiquitous phenomenon, because all tumor cell lines tested (renal, breast, prostate, and bladder carcinoma cell lines and monkey COS7 cells) that expressed FGF-5 and HLA-A3 were recognized by the CTL [35]. A review of the literature identified two types of protein splicing, but neither has been found in vertebrates.

Intein-mediated protein splicing

Intein-mediated protein splicing was first identified in the yeast *TFP1* gene [46, 47] and is defined as the excision of an intervening protein sequence (the intein) from a protein precursor and the concomitant ligation of the flanking protein fragments (the exteins) to form a mature extein host protein and the free intein [48]. More than 115 inteins are registered in InBase http://www.neb.com/neb/inteins.html) from Archaea, Bacteria, and Eukarya but not from higher organisms such as Vertebrata. The intein is a self-excising catalytic unit, and the smallest size for an intein found so

far is 134 aa [49]. Inteins do not require auxiliary factors, and the direct evidence for this was obtained from the following experiment. Xu et al. inserted an intein sequence derived from *Pyrococcus* GB-D DNA polymerase between maltose binding protein (MBP) and paramyosin. Because the protein-splicing reaction is inefficient at low temperature, they could purify the precursor protein using an MBP affinity column, and then they successfully reconstituted the splicing in vitro by shifting to a higher temperature (~65°C) without adding any auxiliary factors [50]. There are four criteria for intein designation:

1. An in-frame insertion in a gene that has a previously sequenced homologue lacking the insertion.
2. Experimental proof of splicing such as an identification of spliced peptides by mass spectromeric analysis.
3. Existence of sequence motifs within the intein.
4. The presence of the four conserved splice-junction residues such as serine, threonine, or cysteine at the intein N-terminus (refer to InBase for more details).

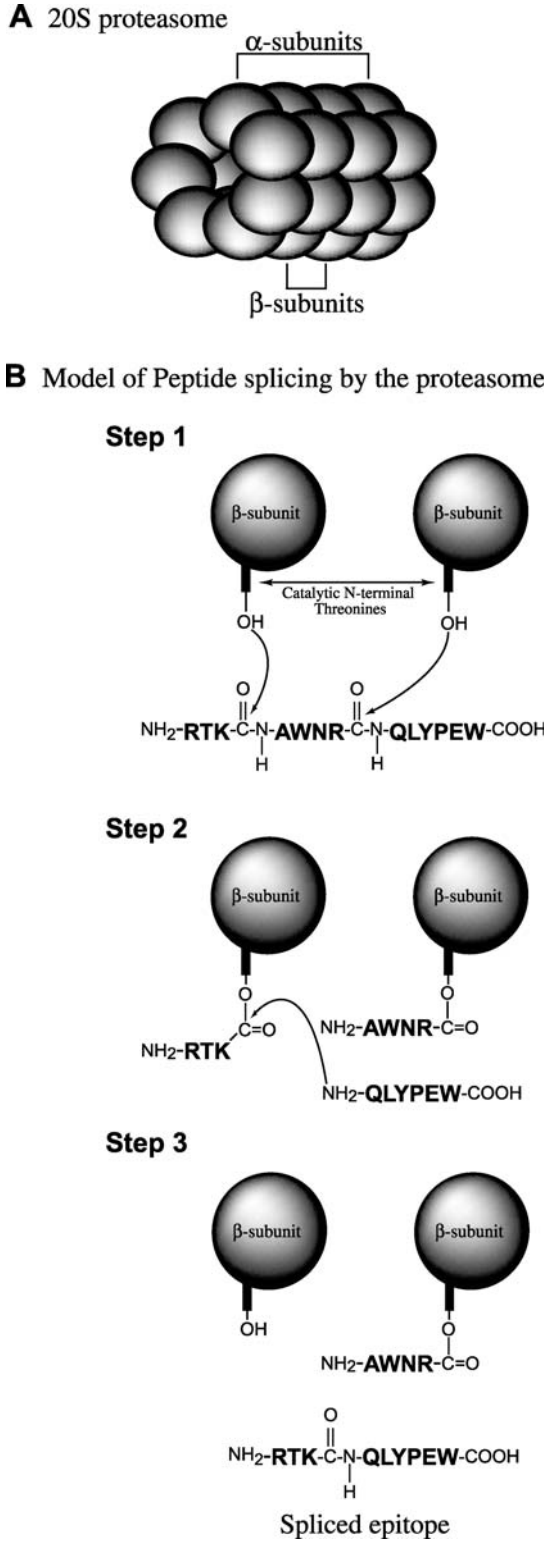
Intein-mediated protein splicing does not seem to explain the mechanism of splicing in FGF-5, because the intervening sequence in native FGF-5 is only 40 aa, and by gene-truncation analysis it was possible to shorten it to as few as 18 aa, while preserving splicing. In addition, none of the intein motifs or splice-junction motifs is present in FGF-5.

Post-translational splicing by reverse proteolysis

Another example of post-translational splicing was discovered in plants and occurs with the lectin concanavalin A (Con A) [51]. In the maturation of jack beans, the initial precursor of Con A (glyco-pro-Con A) is first activated by deglycosylation to pro-Con A. Pro-Con A is then cleaved to produce two distinct proteins that are transposed and re-ligated to become mature Con A. Although the exact mechanism of this splicing in plants is still not clear, one in vitro study showed that asparaginyl endopeptidase can digest Con A and then re-ligate the digested fragments by its reverse proteolytic activity [52]. Reverse proteolysis by an unidentified protease remains a possible mechanism for the splicing of the FGF-5 epitope. For a protein to be cut and re-ligated, the intermediates must be bound to a catalytic unit, compartmentalized, or exist in very high concentration to avoid interference from other competing proteins (Con A can constitute 20% of total jack bean protein [53]). Our data using the proteasome inhibitor *clasto*-lactacystin β-lactone showed that the presentation of the FGF-5 peptide was proteasome dependent, but could not determine if its role was actually in splicing or conventional processing of the spliced form of FGF-5.

Peptide splicing by the human proteasome

Vigneron et al. had isolated a melanoma-reactive CTL clone that recognized antigen $gp100^{PMEL17}$ and were attempting to localize the epitope presented by HLA-A32. Through a similar series of experiments, they eventually determined that the epitope was again post-translationally spliced. Their minimal precursor length obtained by truncation analysis was 13 aa, and the intervening deleted sequence was only four aa. They showed that the proteasome could mediate the splicing event by incubating highly purified proteasomes with the 13-mer precursor peptide (RTKAWNRQLYPEW) in vitro and obtained the spliced 9-mer epitope (RTKQLYPEW) as shown by CTL recognition and mass-spectrometric analysis [54]. The proteasome is a multi-subunit proteinase complex that is composed of the 20S proteasome and the 19S regulatory subunit. The functions of the19S complex include binding polyubiquitin chains [55], catalyzing the release of free ubiquitin [56] and unfolding substrates and transporting them into the 20S subunit in an ATP-dependent process [57]. In the cylindrical 20S proteasome, there are four rings comprised of either seven α subunits or seven β subunits ordered as $\alpha_7\beta_7\beta_7\alpha_7$ (Fig. 3a). The overall dimensions of the cylindrical complex are 148 Å in length and 113 Å in diameter. The entrance in the α subunit is 13 Å in diameter, and only an unfolded peptide can enter [58]. Three out of seven β subunits are known to have catalytic activities. β1 has peptidylglutamyl-peptide hydrolyzing activity, which cleaves after acidic residues [59], β2 has trypsin-like activity, which cleaves after basic residues [60], and β5 has chymotrypsin-like activity cleaving after hydrophobic residues [61]. These subunits are also known to be replaced by LMP2 (β1i), MECL-1 (β2i), or LMP7 (β5i), respectively, in a variant proteasome complex, the immunoproteasome, found in professional antigen-presenting cells or in some IFN-γ treated cells. The function of the remaining β subunits and all the α subunits is still unclear. An N-terminal threonine on each of the three active β subunits catalyzes peptide-bond cleavage, and thus the proteasome belongs to a new mechanistic class of proteases, the N-terminal nucleophile hydrolases [62, 63]. Cleavage by the proteasome is known to occur by nucleophilic attack on the peptide bond by the N-terminal threonine, resulting in the formation of an acyl-enzyme intermediate [58]. The peptide in this intermediate is usually rapidly hydrolyzed and released from the proteasome. However, in the small space within the cylindrical structure of the proteasome, Vigneron et al. hypothesized that the N-terminal fragment of the cleaved peptide might compete with water in a nucleophilic attack on the ester bond of the acyl-intermediate, thereby forming a new peptide bond (Fig. 3b). They provided data supporting this model by showing that mixing purified proteasomes in vitro with RTK and QLYPEW or RTK and AWNRQLYPEW did not generate the epitope (RTKQLYPEW), but RTKAWNR and QLYPEW did (implying the energy for the splice came from the peptide bond between RTK and the excised segment, AWNR, consistent with the proposed acyl-intermediate). In addition, they showed that epitope generation was prevented by the N-terminal acetylation of QLYPEW. Interestingly, D-alanyl-D-alanyl transpeptidases, which have similarity with the proteasome in their catalytically active sites, are known to catalyze the cross-linking of peptidogly-



◀ **Fig. 3** **a** Structure of the 20S proteasome. There are four rings composed of seven subunits in the order of α7β7β7α7. β1, β2, and β5 subunits are known to have the catalytic activity. Because there are two layers of the β-subunit ring, there are six catalytic sites per proteasome molecule. **b** Model of the peptide-splicing reaction proposed by Vigneron et al. [54]. Step 1. Cleavage by the proteasome is by the nucleophilic attack on peptide bond by the catalytic threonines that exist on the amino terminus of β1, β2, and β5 subunits. Step 2. This attack results in the formation of an acyl-enzyme intermediate (in this case, RTK and AWNR bound to β subunits). Usually, these peptides are released from the proteasome by a rapid hydrolyzation. However, in the small compartment within the proteasome, where there are peptide fragments resulting from cleavages, N-terminus of these fragments can compete with water molecules and make a nucleophilic attack on the ester bond of the acyl-enzyme intermediate, forming a new peptide bond and producing the spliced peptide (Step 3). The energy needed for the generation of the new peptide bond seems to be recycled from the bond between RTK and AWNR

cans in bacterial cell walls by transpeptidation via the formation of an acyl-enzyme intermediate [64]. Transpeptidation might be a shared activity of N-terminal nucleophile hydrolases. Vigrenon's model predicted that splicing does not depend on a particular sequence motif but could occur with any fragment produced by the proteasome. In fact, mass spectrometry of the products of the precursor and the proteasome mix showed the predicted alternative fusion peptide RTKAQLYPEW (the proteasome cleaves the precursor RTKAWNRQLYPEW after K, A, and R). Whether FGF-5 is spliced by the same mechanism remains unknown. The lack of impact of varying internal deletions of FGF-5 on splicing suggests such a non-specific mechanism. Proteasome-based splicing requires only that it cleave after NTYAS and before PRFK to generate the epitope NTYASPRFK. One major difference between splicing gp100 and FGF-5 is the length of the intervening sequence, four aa versus 40 aa, respectively. It is estimated that the shortest distance between two active sites in the proteasome is 28 Å, which can be spanned by a hepta- or octapeptide in extended conformation [58]. This mechanism implies a longer-lived acyl-enzyme intermediate, to allow the juxtaposition of this intermediate with PRFK within the narrow confines of the proteasome, but this remains speculative. The proposed mechanism by Vigneron et al. requires both further validation and more complete details prior to acceptance.

Aside from its mechanism, the frequency of post-translational splicing remains to be determined. The original list of peptides eluted from human class I MHC molecules included over 800 peptides with about 200 peptides of unknown origin [65]. Improvements in protein databases since the publication of this list allowed the identification of some peptides from the list [66]. However, the majority of the 200 peptides still remain of unknown origin and some may be generated by splicing. Recent developments have suggested that protein/peptide splicing may not be a rare phenomenon as a third example, for the melanoma-melanocyte antigen tyrosinase, has been found by CTL screening (Dr. Paul Robbins, personal communication). In this finding, two separate peptide sequences are transposed and re-ligated, accompanied by the excision of 27 intervening amino acid residues to form a minimal determinant epitope presented by HLA-A24. This example is, because of the transpositioning, even more complicated than the gp100 and FGF-5 cases. The mechanisms responsible for this transposition and splicing are unknown.

The discovery of protein splicing using the exquisitely sensitive detection modality of the TCR is yet another

example of this methodology uncovering novel biochemical processes in human cells. In 1996, Wang and co-workers, using a CTL clone reactive with the melanoma-melanocyte antigen, gp75 (TRP-1), demonstrated that it recognized a 9-aa epitope in the context of HLA-A31 that was encoded by an alternative open reading frame of the normal *gp75* gene [67]. This alternative ORF encoded a frame-shifted, 24-aa peptide and was apparently expressed by both melanoma cells and normal melanocytes, as both were recognized by the CTL clone. The same group of investigators subsequently found another example of an antigenic epitope arising from an alternative open reading frame for the tumor-testis antigen NY-ESO-1 (expressed by numerous tumor types and in this case, presented in the context of HLA-A31) [68]. This went on to become a common finding with other groups reporting similar scenarios for tumor antigens such as LAGE-1 [69], ICE [70], M-CSF [71], and BING-4 [72]. These examples from epitope identification studies imply that this occurs continuously at low levels in normal as well as transformed human cells, but whether it has functional significance beyond immune recognition is not established.

Another epitope-generating process identified in humans by examining CTL recognition was the translation of proteins entirely or partially from intronic sequences, many due to defective splicing of unmutated gene products. The frequency of this occurrence also seems to be surprisingly high as there are now multiple examples of this phenomenon including TRP-2 [73], GnT-V [74], and gp100 [75]. CTL epitope identification was also the means by which another previously undocumented event in mammalian protein expression was demonstrated. A CTL was found that reacted with a renal carcinoma line as well as with target cells co-transfected with a tumor-derived cDNA and the gene for the restriction element, *HLA-B7*. The tumor-derived cDNA was found to correspond to mRNA arising from the transcription of the antisense strand of the gene for RU2 [76]. Transcription starts at a cryptic promoter in the antisense strand of the first intron of the *RU2* gene and proceeds backwards into the reverse strand of the normal *RU2* promoter where, fortuitously, there is a chance polyadenylation signal. The incomplete overlap of the two complementary messages allowed the measurement of expression of the two transcripts by RT-PCR. The "sense" transcript was found in all normal tissues, whereas the antisense transcript was well expressed in a broad array of tumors and at modest levels in normal kidney, liver, testis, and bladder [76].

Conclusion

Our quest to define tumor-associated antigens recognized by T-cells resulted in the unexpected discovery of post-translational splicing in humans. At the moment, there are many questions remaining unanswered. How common is this? Is there differential splicing in various normal and malignant cells? Are the spliced peptides regarded as self or foreign by the immune system? If the splicing happens randomly between the fragments generated by the proteasome, what is the definition of the immunological self? Are there spliced variants that survive the proteasome and have independent functional roles?

Now that immunologists know that epitopes are not necessarily predictable from primary protein sequences, there will likely be more examples of post-translationally spliced epitopes. These new examples may lead us to answers to these questions.

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