Probing proteasome activity and function : cancer diagnostics and mechanism of antigen processing

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Chapter 3.4

Summary and prospects: proteasome splicing

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Following malignant transformation or viral infection, a broad repertoire of antigenic peptides is presented on the cell surface by MHC class I, enabling CD8+ T cells to sense the change in intracellular protein content, which eventually leads to killing of the antigen presenting cell. The 26S proteasome is responsible for the generation of these antigenic peptides *in vivo*. Recently, it has become apparent that not only contiguous antigens, but also non-contiguous antigens, that consist of two post-translationally fused peptides, can be presented to the immune system. The ligation that produces these epitopes is thought to be catalyzed by the proteasome *in vivo* via a transpeptidation mechanism, which makes it likely that splicing is not a random event, but governed by distinct splicing rules. The elucidation of such splicing rules will facilitate the prediction and detection of spliced antigens, which will enable studying the roles of such spliced epitopes in immunity.

To be able to study proteasome splicing on a molecular level, large amounts of purified proteasome are required. In **chapter 2.3** we described how the fluorescent proteasome activity probes described in chapter 1 can be used to monitor proteasome purity during large scale proteasome isolation from bovine liver and spleen. Using mass spectrometry, we subsequently showed that highly purified preparations of either constitutive or immunoproteasome could be obtained via this method. In **chapter 3.2**, we used these proteasome preparations to decipher splicing rules. By analyzing proteasome digestion mixtures of small libraries of potential ligation partners, we were able to show that splicing motifs exist. Subsequently, we confirmed and refined these motifs by application to a polypeptide derived from the Influenza A neuraminidase 1 protein. In addition, we showed that proteasomal splicing reactions can occur with high efficiencies of up to 30% *in vitro* if structural requirements for proteasome splicing are met by both ligation partners and that many spliced epitopes may be formed from a single protein. Finally, we examined the mechanism of splicing reactions, and showed that *in vivo,* intrastrand splicing of peptides derived from the same protein is likely preferred over random ligation of peptides derived from different proteins. In **chapter 3.3**, we investigated in more detail a specific type of proteasomal ligation reaction, in which the C-terminal ligation partner has lysine at the site of ligation. As lysine has two amino groups that can theoretically both participate in proteasomal transpeptidation reactions, this implies that the proteasome may be able to from isopeptide linkages. Using NMR, we showed that both the α- and the ε-amino groups of lysine were able to participate in ligation reactions, and we could determine that ε-ligation was favored over α-ligation in 10% of all splicing reactions involving lysine at the site of ligation. This suggested that the proteasome can create isopeptide linkages and may therefore form a novel type of antigen that may play a role in immunity *in vivo*. In addition, we showed that isopeptide linkage containing epitopes have unique properties that discern them from normal epitopes. First, isopeptides

of various lengths can bind to HLA-A2.1 and HLA-A3 proteins with high affinity. Second, isopeptides are more stable towards further proteasomal processing as compared to normal peptides. The combination of these properties suggests that a large fraction of isopeptide epitopes may reach the cell surface to evoke an immune response.

The data described in **chapters 3.2 and 3.3** collectively indicate that different types of proteasomal ligation reactions readily occur *in vitro*. The efficiencies of these reactions are high compared to the estimated ligation efficiencies of splicing reactions described *in vivo*, suggesting that proteasomal splicing reactions may occur more frequently than assumed thus far and may significantly contribute to immunity *in vivo*. To date, only three spliced epitopes have been described *in vivo*, as identifying spliced antigens *de novo* is not possible with the methodologies currently used to identify novel antigens. Novel epitopes can be identified by cell surface elution of all self- and foreign epitopes, followed by MS analysis and matching against protein databases. Alternatively, epitopes may be predicted from protein sequences and tested for T cell receptor recognition, e.g. by incorporation into fluorescent MHC class I tetramers, which are subsequently used to stain T cells in Fluorescence assisted cell sorting (FACS) assays. However, as spliced antigens are not contiguous and their formation requires post-translational splicing, they neither match existing databases nor can they be predicted from contiguous protein sequences, which hampers their identification. The splicing rules deduced in chapter 3.2 should facilitate the identification of more spliced epitopes, and several types of experiments that aim at identifying both normal and $^{\varepsilon}$K linked spliced antigens are currently ongoing.

To show that our splicing rules can predict splicing *in vivo*, we have expressed optimized precursor peptides in cells. If the predicted proteasomal splicing reactions occur, spliced epitopes are formed, loaded onto MHC class I and transported to the cell surface, where they can be identified using two strategies. The first strategy is based on elution of all epitopes from the cell surface, followed by LC-MS analysis. The successful identification of spliced epitopes requires the ability to detect and sequence low femtomolar amounts of individual peptides in highly complex mixtures eluted from the cell surface. Typically, the number of copies of a particular peptide presented by MHC molecules on a single cell ranges from 1 to 10.000, and therefore, MS identification of spliced epitopes is very challenging. Very low copy numbers of MHC-presented epitopes can already activate CD8+ T cells, and T cell activation assays can therefore also be used to identify spliced epitopes. To perform these assays, mice transgenic for HLA-A2 (A2-Kb) are first vaccinated with the anticipated splicing product to obtain specific CD8+ T cells. These T cells are subsequently added to the cells that are transfected with an optimized precursor peptide *in vitro*. The presence of the expected splicing product on the cell surface will activate the T cells, which can be monitored in interferon-γ release assays. Although T cell activation assays are very sensitive, the expected spliced product has to be immunogenic to evoke a T cell response, which may complicate the identification of predicted spliced products. Finally, vaccination experiments are currently ongoing to show that splicing occurs readily *in vivo*. In these experiments, mice are first vaccinated with a DNA vaccine encoding splicing precursors. At the top of the immune response, blood is analyzed for the presence of T cells that are reactive against the spliced product.

Identifying isopeptide linkage containing epitopes *in vivo* is even more challenging, as mass spectrometry cannot distinguish between N^{ε}- and N^{α}-linked lysine residues. Isopeptide epitopes have unique properties that can be used to distinguish them from normal peptides, but the amounts of epitope that are eluted from the surface of antigen presenting cells is generally not sufficient to perform secondary assays. Therefore, only T cell activation assays can be used to identify this novel type of antigen and we are currently in the process of peptide vaccinating HLA-A2 transgenic mice with both normal epitopes and isopeptide epitopes to generate T cells that are specific for either normal or isopeptide epitopes. By using these T cells in combination with cells that are transfected with an optimized splicing precursor peptide, we hope to identify isopeptide epitopes *in vivo*

Finally, studies are currently ongoing that will further refine the splicing rules that are postulated in this thesis. Application of the methods developed in chapter 3.2 to immunoproteasomal digestion mixtures will likely generate a set of splicing rules for the immunoproteasome. In addition, knowledge of proteasomal cleavage sites will allow a more accurate prediction of both C- and N-terminal ligation partners. Algorithms that predict proteasomal cleavage are available, but they did not accurately predict hydrolysis of our model peptides. By analyzing a larger set of splicing precursors, rules that predict proteasomal cleavage can likely be deduced. We expect that the studies presented in this thesis will aid the discovery of novel spliced epitopes and that they will contribute to unraveling the role of spliced epitopes and isopeptide antigens *in vivo*.