Activity-based proteasome profiling

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General Introduction

1.1 The ubiquitin proteasome system and the activity of the proteasome

The ubiquitin proteasome system (UPS) is the central protein turnover machinery in eukaryotic cells(*1*). Proteins that are at the end of their lifecycle are labeled by a small protein post translational modification (PTM), about 8 KDa, called ubiquitin. Protein substrates are modified on their lysine residues by ubiquitin through its C terminus, under the catalysis of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). Following addition of a single ubiquitin moiety to a protein substrate, further ubiquitin molecules can be added to the first, yielding a polyubiquitin chain(*2*). The polyubiquitin chain is recognized as the signal for proteasomal degradation of the substrate, also known as the "kiss of death". The ubiquitin molecules are removed from the protein substrates by deubiquitinating enzymes and recycled for ubiquitinylation of new substrates (Fig 1).

Polyubiquitinylated proteins can be transferred by ubiquitin adapter/receptor proteins to the proteasome for controlled degradation(*2, 3*). Ubiquitin receptor proteins recognize polyubiquitinylated proteins by their UBA (ubiquitin associate) domains, and transport them to the proteasome through the binding by UBL (ubiquitin like) domains to subunits of the 19S proteasome. Ubiquitin receptor proteins Rad23 and Dsk2 do not get degraded by the proteasome due to their special sequences and have long lifetime(*4*).

The proteasome is a multi-subunit protease cluster, which is evolutionarily conserved. In mammalian cells the proteolytically active forms of proteasome are the 26S or 30S proteasomes(*1, 5*). Structurally, the 26S proteasome contains one barrel like shaped 20S core particle and one 19S regulatory particle on one side of the 20S barrel, while 30S contains two 19S particles on both sides of the 20S. The 19S particles recognize polyubiquitinylated proteins, remove the polyubiquitin chains from the substrates, unfold the substrates and open the gate of the 20S particle(*6*).

Linearized polypeptides are degraded by the 20S core particles into peptides of about 4-25 amino acids(*7*). These peptides can be further degraded by downstream amino-

peptidases into single amino acids. The 20S particle has a barrel like shape, involving four hetero heptameric subunit rings, two identical β rings in the middle and two α rings on the outside. Among seven different α subunits (α1-α7) and seven different β subunits (β1-β7), 3 subunits β1, β2, β5 have proteolytic activity. The three active subunits have distinguished cleavage preferences. Generally, β1 cleaves at the C terminus of acidic amino acids; β2 cleaves after basic amino acids; and β5 cleaves after hydrophobic amino acids(*5*, *8*). This kind of difference makes the proteasome capable of cutting most of the poly-peptide sequences. The difference mostly results from their different sequences and shapes of the binding pockets. The catalytic amino acid residue in the active proteasome subunits is the N-terminal threonine.

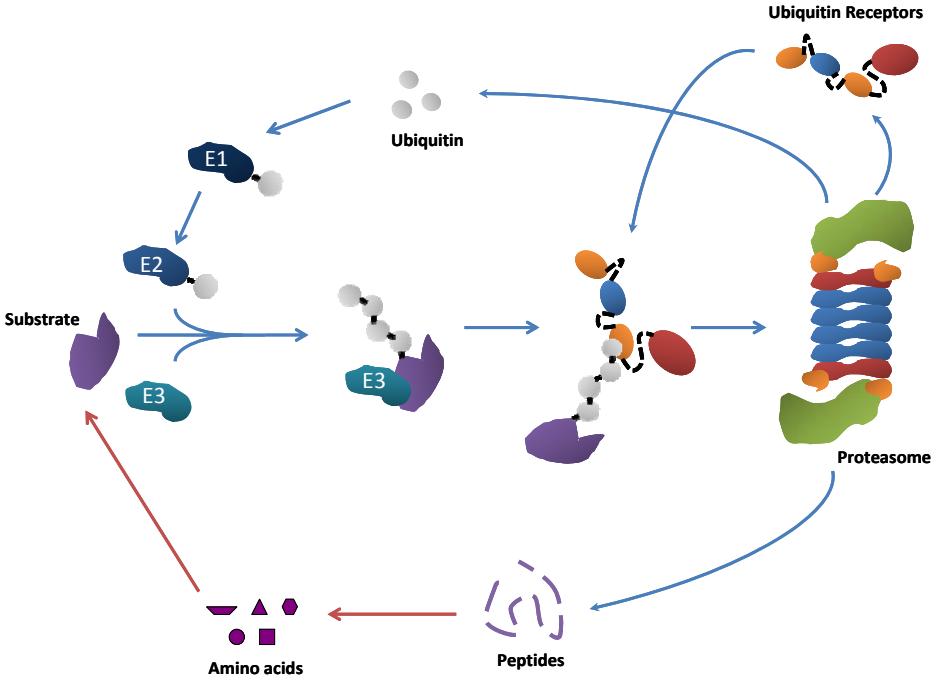

Figure 1: General scheme of the Ubiquitin-Proteasome system. Proteins (substrates) are modified by ubiquitin under catalysis of E1, E2 and E3 ubiquitin ligases and then transported by ubiquitin receptors to the proteasome for controlled degradation. The ubiquitin molecules are recycled for labeling of new substrates. Peptides resulted from proteasome degradation are hydrolyzed into amino acids. The amino acids are used for synthesis of new proteins.

Vertebrates have developed immune system, in which proteasomes play an important role. Peptides resulting from proteasomal degradation can be processed and presented as antigenic peptides on the cell surface by MHC (major histocompatibility complex) I and verified by CD8+ T cells(*9*, *10*). Besides constitutive proteasome, immune cells express a second type of 20S proteasome, known as immuno proteasome. In the

immuno proteasome, the active subunits β1, β2, β5 are replaced for another three active subunits β1i, β2i, β5i, which show slightly different substrate preferences with their constitutive homologues(*11*).

In vertebrates, a third type of 20S particle was identified recently as thymo proteasome for its excluding existence in the cortical thymus epithelial cells (cTEC)(*12*). In the thymo proteasome, a different active subunit β5t replaces the β5i in the immuno proteasome to form the new particle. β5t-deficient mice showed less efficient positive selection of CD8+ T cells(*13*).

1.2 Activity-based proteasome profiling

The activity-based protein profiling (ABPP) is applied to determine proteasome activity specifically in complex biological systems, for instance a cell lysate or living cells(*14, 15*). Synthetic, covalent and irreversible enzyme inhibitors that are modified with reporter groups like fluorophores or biotin, named activity-based probes (ABP), are used to label the active enzymes in the ABPP experiments. The probe-enzyme reaction ensures that only the catalytically active enzyme is targeted by the ABP.

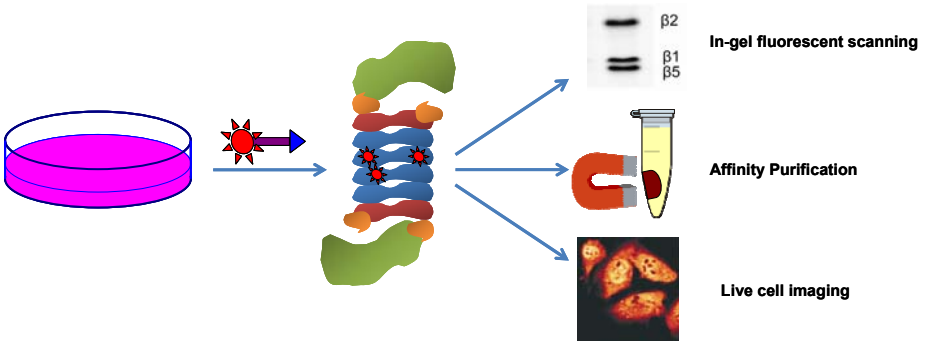

Figure 2: Schematic presentation of the application of several activity-based proteasome profiling approaches. Proteasomes are labeled by Activity-based probes in living cells and the fluorescent probe labeled proteasome active subunits are resolved by SDS-PAGE and visualized by in-gel fluorescent scanning. Affinity purification is used to enrich the active proteasome subunits that are labeled by biotinylated ABP. Fluorescent proteasome ABPs can also be used for live cell imaging to monitor the proteasome localization in the living cells by fluorescent microscopy.

A typical proteasome ABP involves three parts, the warhead, the linker and the tag(*14, 16*). The warhead is normally an electrophilic trap which can react with the active N-terminal threonine of the active proteasome subunits, for instance the epoxyketone or vinyl sulphone groups(*17*). The linker is generally a short peptide which can be recognized by the proteasome subunits as their endogenous substrates. The tag can be either a fluorophore or a biotin group, for visualization by in gel fluorescent scanning or biotin-streptavidin

immuno blot(*18*). The fluorescent group can also be used for live cell imaging by fluorescent microscopy, or staining of the active proteasome in FACS (fluorescence-activated cell sorting) experiments(*19, 20*). Biotin-streptavidin affinity purification is used to enrich the active proteasome subunits for protein identification or quantification (Fig 2). In some cases, the specificity and cell permeability of the probes can be altered by chemical groups like fluorophores or biotin. To circumvent these effects, an azide or alkyne or tetrazin group can be installed on the probes instead of the tag. After the first step labeling of the proteasome with the probes, the tags can be installed onto the probes by bioorthogonal chemistry(*21, 22*).

Active proteasome subunits were identified as the binding target of Streptomyces metabolite lactacystin using its radioactive ^{3}H-labeled derivatives, in murine neuroblast cells(*23*). The anti-tumor natural product epoxomicin was shown to be a proteasome inhibitor by labeling active proteasome subunits with biotin-epoxomicin(*24*).Since that time, several generations of activity-based proteasome probes have been developed, broad spectrum and subunit specific(*17*). For instance, ^{125}I-labeled peptide vinyl sulfone L_3VS (^{125}I-NIP-L_3VS) and the N-terminal extended biotinylated AdaK(bio)Ahx$_3$L$_3$VS derivative were applied to reveal the proteasome activity in either bacteria or EL4 murine T cells(*25, 26*). DansylAhx$_3$L$_3$VS was synthesized for in vivo profiling of the specificity of proteasome inhibitor bortezomib via dansyl fluorescence or western blotting detection with antibodies against dansyl(*27*). The pan-reactive MV151 equipped with lighter bodipy fluorophore was used for revealing proteasome activity in bortezomib adapted myeloma cells(*28*).

1.3 Aim and outlines of this thesis

The work described in this thesis is mainly focusing on setting up and application of a quantitative activity-based proteasome profiling method.

Chapter 1 provides a general introduction on the ubiquitin proteasome system (UPS) and activity-based proteasome profiling.

Chapter 2 is a literature review of some new achievements in the activity-based protein profiling field in the recent years, focusing on application in biochemistry, molecular and cellular biology, medicinal chemistry, pathology, physiology and pharmacology research.

Chapter 3 is a protocol for performing quantitative activity-based proteasome profiling experiments. In the protocol, both high throughput fluorescent ABPP and biotinylated probe plus LC/MS approaches are described.

Chapter 4 is a brief technical report about bioorthogonal chemistry in ABPP. The commonly used secondary azide group is compared with a primary azide group in proteasome ABPs performing Cu(I) catalyzed azide-alkyne cycloaddition and Staudinger-Bertozzi reaction under native/denatured protein conditions.

Chapter 5 is focusing on the application of quantitative activity-based proteasome profiling in the prognosis of cancer therapeutics. A combination of ABPP and global proteomics is performed to elucidate the bortezomib sensitivity and resistance mechanisms in leukemia and solid tumor cells.

Chapter 6 describes the characterization of the newly discovered proteasome subunit β5t by ABPP and LC/MS proteomics. The subunit is proven to be catalytically active. A hydrophilic Thr residue on the P2 position of the proteasome inhibitor improves the inhibitory efficiency of β5t, which indicates it might prefer to cleave hydrophilic peptides.

Chapter 7 describes the identification of O-GlcNAcylation modifications on the ubiquitin receptor protein hHR23B and characterization of how the sugar moiety influences the conformation and functions of the protein.

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