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Brief Communication

Activity-Based Profiling Reveals Reactivity of the Murine Thymoproteasome-Specific Subunit β5t

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SUMMARY

Epithelial cells of the thymus cortex express a unique proteasome particle involved in positive T cell selection. This thymoproteasome contains the recently discovered β5t subunit that has an uncharted activity, if any. We synthesized fluorescent epoxomicin probes that were used in a chemical proteomics approach, entailing activity-based profiling, affinity purification, and LC-MS identification, to demonstrate that the β5t subunit is catalytically active in the murine thymus. A panel of established proteasome inhibitors showed that the broad-spectrum inhibitor epoxomicin blocks the β5t activity and that the subunit-specific antagonists bortezomib and NC005 do not inhibit β5t. We show that β5t has a substrate preference distinct from β5/β5i that might explain how the thymoproteasome generates the MHC class I peptide repertoire needed for positive T cell selection.

INTRODUCTION

The ability to recognize nonself oligopeptides is a key feature of mammalian immunity. T cells that recognize antigenic oligopeptides elicit a directed adaptive immune response aimed at the identification and eventual eradication of the invading pathogen that is the source of the nonself protein from which the antigenic oligopeptide is derived (Janeway and Bottomly, 1994; Medzhitov, 2007). T cell recognition is effected by binding of specific T cell receptors to the antigenic peptides that are complexed to either major hisocompatibility complex (MHC) class I or MHC class II molecules (Huseby et al., 2005; Takahama et al., 2008). MHC I molecules present oligopeptides derived from cytosolic and nuclear proteins to $CD8^+$ cytotoxic T lymphocytes (CTL) and by this virtue report on the presence of virally encoded proteins (Kloetzel and Ossendorp, 2004). T cells specific for nonself peptides are produced by thymic selection. The generation in the thymus of nonself peptide-selective CTL proceeds in two discreet events (Nitta et al., 2008). Positive selection is mediated by cortical thymic epithelial cells. In this process, thymocytes expressing T cell receptors are confronted with tissues expressing MHC I molecules loaded with oligopeptides. Current understanding is that the MHC I/peptide antigen complexes produced by cortical thymic epithelial cells are low-affinity T cell receptor binders. Thymocytes passing through the thymic cortex that bind to MHC I molecules carrying a peptide load are selected from thymocytes expressing nonbinding receptors. In the ensuing negative selection step, mediated by medullary thymic epithelial cells, thymocytes from the positively selected pool that are responsive to MHC I molecules exposing self-peptides are eliminated.

Recently, Tanaka and co-workers made a major breakthrough toward understanding how positive selection proceeds (Murata et al., 2007). They found that epithelial cells at the thymic cortex express, next to the constitutive proteasome and the immunoproteasome, a third 20S proteasome particle which was dubbed the thymoproteasome. The 20S core particle of the proteasome is assembled from α and β subunits in a pattern of four, stacked, heptameric rings (α1-7, β1-7, β1-7, α1-7) generating a barrel-shaped structure that contains two copies of the catalytically active β subunits: β1 (post acidic), β2 (tryptic-like), β5 (chymotriptic-like) peptidase activities (Baumeister et al., 1998). The thymoproteasome contains the β1i and β2i subunits just like the immunoproteasome, with the important exception that the unique subunit β5t replaces the immunoproteasome-specific subunit, β5i.

The thymoproteasome is the most abundant proteasome species in cortical thymic epithelial cells (cTEC). Thymoproteasome expression may have implications for the repertoire of oligopeptides presented by MHC I molecules on the surface of cTECs that might significantly differ from to the repertoire produced by medullary thymic epithelial cells. Closer inspection of the thymoproteasome 20S particle revealed that, in contrast to the constitutive and the immunoproteasome, it possessed little chymotryptic activity, a finding that seems to correlate

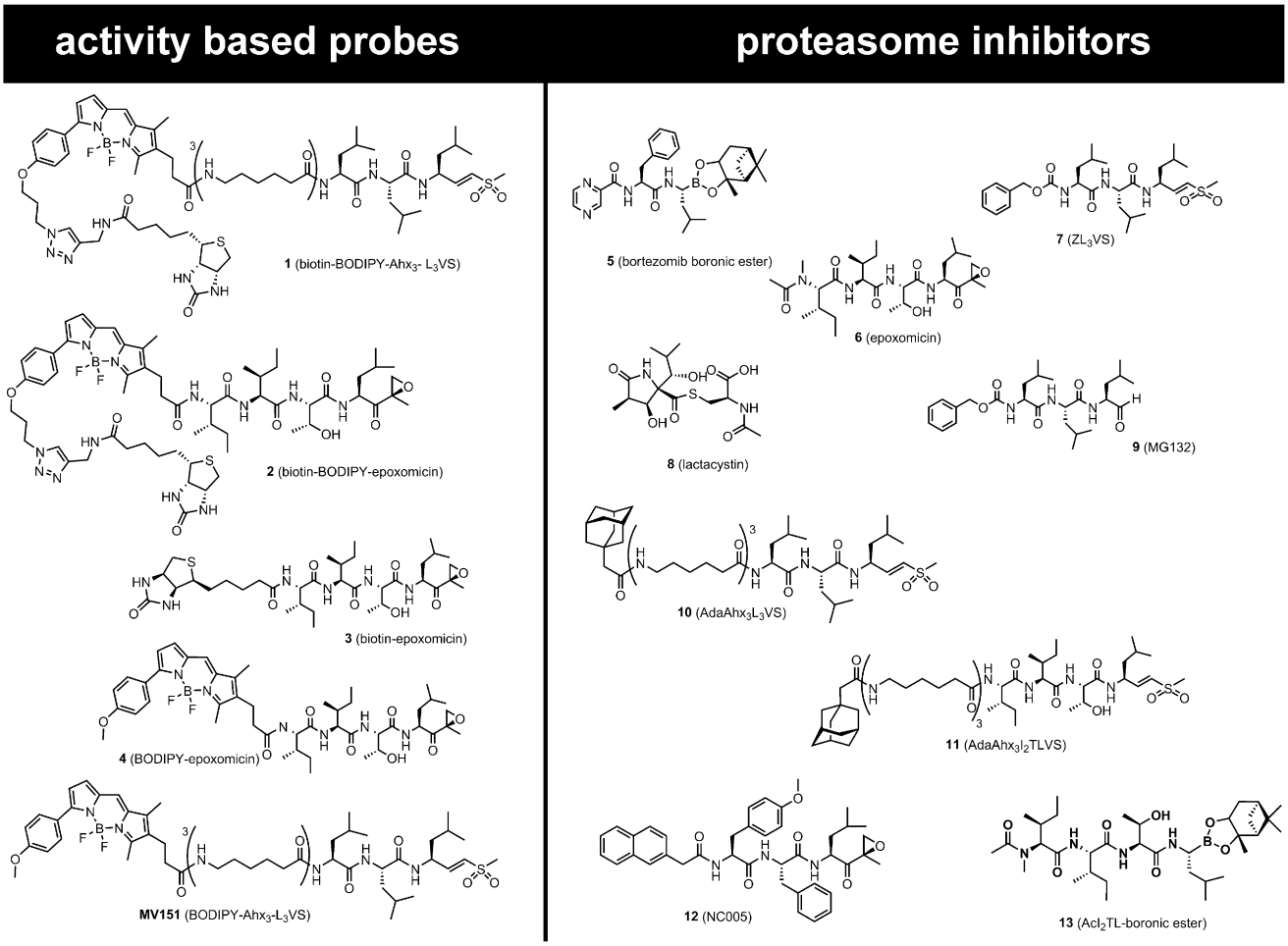

Figure 1. Activity-Based Probes and Proteasome Inhibitors Used in This Study

In addition to the enzyme reactive group (warhead) and targeting sequence of the inhibitors, activity-based probes are equipped with a fluorophore for in-gel detection, a biotin tag for affinity purification or with both.

with the hydrophilic nature of the putative substrate-binding site of β5t compared with β5/β5i (Murata et al., 2007). In theory, β5t can contribute in two ways to the generation of specific MHC I peptides used in positive T cell selection (Murata et al., 2008). It could act as an impassive, catalytically inactive bystander, in which case β1i/β2i produces the majority of MHC I peptides with a bias toward their substrate preferences. Alternatively, it could actively participate in protein degradation and assist in producing "nonself" peptides thanks to its intrinsic substrate preference, which then must be distinct from that of β5/β5i.

Activity-based probes are synthetic compounds bearing a reporter or affinity tag and an enzyme reactive group that can covalently bind to the active site of an enzyme (Cravatt et al., 2008). The tagged enzymatic activities can than be visualized by fluorescence or affinity purified, digested with trypsin, and identified by LC/MS analysis. We here demonstrate, by making use of activity-based proteasome probes (Verdoes et al., 2009), that β5t is in fact a catalytically active subunit and show that its preference toward established proteasome inhibitors differs substantially from those of β5/β5i.

RESULTS AND DISCUSSION

Activity-Based Profiling Reveals β5t Activity

As the first experiment, we incubated whole tissue thymus homogenate from 3-week-old mice with the fluorescent broad-spectrum ABPs **1**, (Verdoes et al., 2008) **2**, **4,** and MV151 (Verdoes et al., 2006) shown in Figure 1 (for the synthesis of probes **2** and **4,** see Supplemental Experimental Procedures available online). Proteins were resolved by SDS-PAGE under reducing conditions and fluorescently labeled proteasome subunits were visualized by in-gel fluorescence scanning. In Figure 2A, MV151 shows the typical band pattern of staining that is similar to that of the EL4 cell line expressing the constitutive and the immunoproteasome (Kessler et al., 2001) (Figure S1) indicating that both particles are expressed in the thymus. Peptide vinyl sulphone **1**, the biotinylated derivative of MV151, shows a similar pattern as MV151. Interestingly, the peptide epoxyketones **2** and **4** show two new bands that run below and above the constitutive and immunoproteasome subunits. Of these, the lower band corresponds to β1i.