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O-GlcNAc Peptide Epoxyketones Are Recognized by Mammalian Proteasomes

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Proteasomes are multicatalytic proteinase complexes responsible for the degradation of cytosolic and nuclear proteins. In eukaryotes, proteasome substrates are normally marked for degradation through the attachment of ubiquitin chains at specific sites, and subsequent proteasome-mediated degradation takes place in a controlled and ATP-dependent fashion.[1] In mammals, some oligopeptides generated by proteasomes are presented to the immune surveillance system by major histocompatibility complex class I (MHC I) molecules.[2] Next to self-peptides, peptides derived from virally encoded proteins are also exposed at the cell surface in this fashion, and thus, mammalian proteasomes partake in combating viral infections. Mammals express several proteasome particles that appear to play specific roles in MHC I-mediated immune response. These are the constitutively expressed 26S proteasome, the immunoproteasome, and the recently discovered thymoproteasome. Of these, the latter two are expressed by the specific tissues where they exist next to the 26S proteasome.[3] In all mammalian proteasomes, the actual proteolytic activities reside in the core 20S particles, which are C_2-symmetric barrels composed of two rings of seven distinct β-subunits ($\beta1-\beta7$) sandwiched between two rings composed of seven distinct α-subunits (α1−α7).[4] In 26S proteasomes, $\beta1$, $\beta2$, and $\beta5$ are catalytically active and cleave preferentially after acidic, basic, and hydrophobic residues, respectively.[5] These subunits are replaced by $\beta1$i, $\beta2$i, and $\beta5$i in immunoproteasomes and by $\beta1$i, $\beta2$i, and $\beta5$t in the thymoproteasome. The exact role and substrate preference of the combined seven catalytic subunits is the subject of extensive studies, and the same holds true for elucidating the nature of the peptides produced by proteasomes and recruited by the immune system. In this context, the immunogenicity of MHC I-associated phosphopeptides is well-established, and this implies the involvement of proteasomes in the turnover of phosphorylated proteins.[6]

In the past decades, another major post-translational modification that occurs in the cytoplasm and the nucleus, namely, protein Ser/Thr O-GlcNAcylation, has drawn considerable attention.[7] O-GlcNAcylation is a reversible process, in that O-GlcNAc transferase (OGT) glycosylates a serine or threonine residue and a hexosaminidase (O-GlcNAcase) is responsible for the removal of O-GlcNAc residues. Protein O-GlcNAcylation and proteasomal degradation occur in the same cellular compartments, and it is therefore not surprising that the two processes interface. O-GlcNAcylation of the proteasome down-regulates its activity, whereas O-GlcNAcylation of certain proteins appears to give some protection against proteasomal degradation.[8] At the same time, O-GlcNAcylated MHC I-derived peptides were evidenced in several studies, indicating that O-GlcNAcylated proteins can act as viable proteasome substrates.[9] In regard to whether the proteasome is capable of degrading O-GlcNAcylated proteins, we felt that activity-based profiling using tailored O-GlcNAcylated proteasome inhibitors would give insight into this uncharted matter in a straightforward fashion. To this end, we prepared (Figure 1) a set of four peptide epoxyketones based on the selective proteasome inhibitor epoxomicin[10] and extended with an N-terminal GlcNAcSer derivative. In compounds **1** and **2**, the *N*-acetyl group of glucosamine is replaced by a *N*-(azidoacetyl) group, and the pentapeptide is N-terminally acetylated. Compounds **3** and **4** differ in that the azidoacetyl group is placed at the N-terminus of the peptide and the glucosamine nitrogen is acetylated. Both peracetylated (**1** and **3**) and unprotected (**2** and **4**) derivatives were prepared following the block-coupling strategy we routinely use for the construction of C-terminally modified oligopeptide proteasome inhibitors.[11]

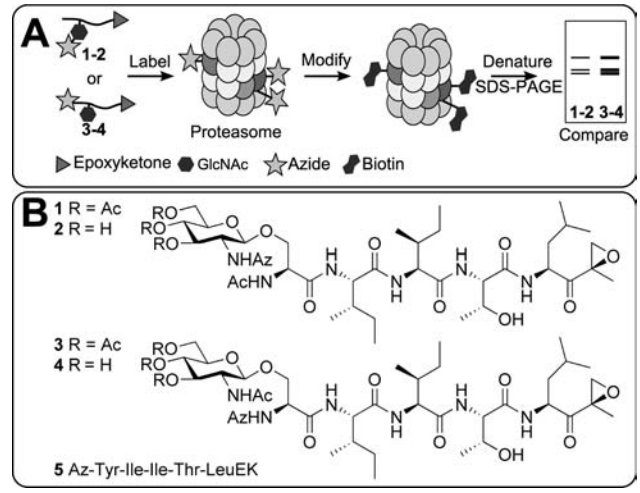

Figure 1. (A) Schematic representation of the experiment. (B) Structure of GlcNAcylated proteasome probes **1**−**4** and control probe **5.** Az = Azidoacetyl, EK = Epoxyketone.

The ability of peptide epoxyketones **1**−**4** to inhibit the proteasome was assessed indirectly in a competition experiment using the pan-reactive and fluorescent proteasome activity-based probe MV151 as a read-out.[12] To this end, lysate derived from HEK cells (a human hybridoma cell line expressing the constitutive proteasome) was treated with increased peptide epoxyketone concentrations prior to treatment with MV151, and the proteins were separated by SDS-PAGE. The fluorescence read-out (Figure 2A, panels 1−4) revealed that the four probes are inhibitors having comparable potencies and show a slight preference for the $\beta5$ subunit with respect to the individual catalytic activities. In all instances, labeling with MV151 is largely abolished at 10 μM final concentration. In the key experiment, samples of cell lysate exposed to **1**−**4** at the same final concentrations were treated with biotinylated phosphine.[11] In this way, proteasome subunits modified with an azide are directly visualized by Staudinger−Bertozzi bioorthogonal labeling[13] followed by SDS-PAGE and Western blotting. The resulting images (Figure 2B, panels 1−4) reveal a

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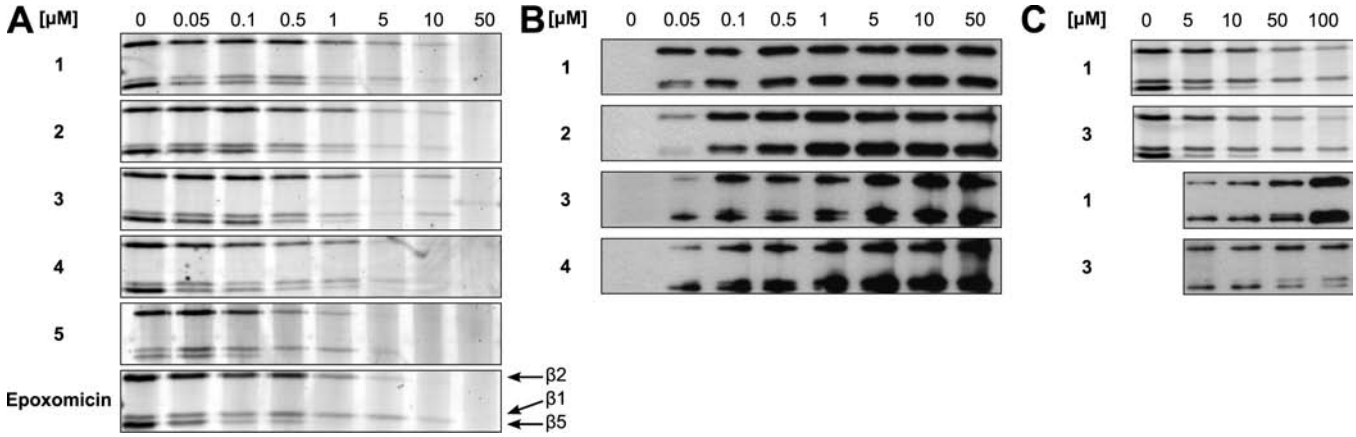

Figure 2. Competition and labeling studies of proteasome probes **1**−**5**. (A) Lysate from HEK293T cells (10 μg of total protein) was incubated with increased concentrations of **1**−**5** and epoxomicin as a control for 1 h at 37 °C. Residual proteasomal activity was labeled by incubation with MV151 (1 μM final concentration) for 1 h at 37 °C. After denaturation and resolution by SDS-PAGE, the potency was determined using fluorescence scanning. (B) Streptavidin blot of HEK293T lysate (10 μg of total protein) treated with increased concentrations of **1**−**4** for 1 h at 37 °C, after which the azide was modified by incubation with the Staudinger−Bertozzi reagent (400 μM final concentration) for 1 h at 37 °C. (C) Labeling of the probe in living HEK293T cells. Some 1×10^6 HEK cells were incubated with the indicated amounts of probes **1** and **3** for 16 h, after which the cells were harvested, lysed, and treated with either MV151 (upper panels) or the Staudinger−Bertozzi reagent (lower panels).

signal that is highly complementary to the one witnessed in panels1−4 of Figure 2A. The disappearance of MV151 labeling is counterbalanced by the appearance of the streptavidin-horseradish peroxidase-mediated signal, and the latter can only be the result of covalent azide introduction onto the proteasome active sites. This is the expected result for compounds **3** and **4**, which have the azide in the peptide backbone (indeed, these results corroborate prior work from our laboratory).[14] Peptide epoxyketones **1** and **2**, however, give equally efficient Staudinger−Bertozzi labeling. We conclude that hexosaminidase-mediated removal of the GlcNAz moiety is not a prerequisite for proteasomal entry and active-site modification by these activity-based probes. In fact, concomitant treatment of lysate incubated with **1** or **2** with hexosaminidase inhibitor NAG-thiazoline[15] did not give a discernibly different outcome.[11] Perhaps surprisingly, proteasomes not only bind O-GlcNAcylated peptide epoxyketones but do so with a potency only slightly lower than that of the parent compound epoxomicin (Figure 2A, panel 6). To establish whether the steric bulk represented by the GlcNAc moieties in **1**−**4** causes this slight loss in inhibitory potential or if specific structural features play a role, we prepared and assessed tyrosine derivative **5**. This compound actually proved the most potent of the series (Figure 2A, panel 5), leading to the tentative conclusion that GlcNAc moieties are accepted by the proteasome. Blocking proteasome activity in live cells necessitated increased concentrations of **1** or **3** but otherwise gave essentially the same picture, demonstrating the use of our probes also in this physiologically more relevant research setting (Figure 2C). Our results thus strongly point toward a natural role for proteasomes in producing Ser/Thr O-GlcNAcylated peptides, some of which may end up on the cell surface as part of MHC I complexes. Whether such complexes are immunologically relevant in health and disease remains an open question. Future research in our laboratory is aimed in this direction through the generation of Ser/Thr O-GlcNAcylated peptide epoxyketones more closely resembling actual proteasome products/MHC I peptides.

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Supporting Information Available: Synthesis and characterization of compounds **1**−**5**, biological assays, structures of MV151 and biotinylated phosphine, and complete ref 12. This material is available free of charge via the Internet at http://pubs.acs.org.

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