Communication

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Periodate-Triggered Cross-Linking Reveals Sug2/Rpt4 as the Molecular Target of a Peptoid Inhibitor of the 19S Proteasome Regulatory Particle

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The 26S proteasome is responsible for most non-lysosomal protein degradation in eukaryotic cells.[1−4] It comprises two large subunits: a barrel-shaped 20S core particle (CP) that contains the proteolytic sites and the 19S regulatory particle (RP) that caps each end of the 20S barrel. The 19S RP includes a ring of six ATPases at its base that perform at least three critical functions. They are involved in binding the polyubiquitin chain attached to many proteasome substrates, unfold substrate proteins, and feed the resultant unstructured chain into the catalytic maw of the 20S CP. Finally, they hold open a "flap" comprising residues from 20S proteins that otherwise blocks access of the substrate to the internal cavity of the 20S CP. Pharmacological inhibitors of the proteolytic activities of the 20S CP are used widely, and one of these compounds is now employed clinically against multiple myeloma.[5,6] However, much less work has been done to manipulate the activity of the 19S RP pharmacologically. In addition to being of potential utility as proteasome inhibitors, these would be interesting tools for probing the many recently discovered non-proteolytic roles of this subcomplex in transcription and DNA repair.[7−12]

We recently reported the first 19S RP inhibitor, RIP-1 (*R*egulatory Particle *I*nhibitor *P*eptoid-1) (Figure 1).[13] RIP-1 was isolated from a combinatorial library of approximately 33 000 peptoids, all of which were capped with a purine molecule in an attempt to bias the library toward binding one or more of the six ATPases in the 19S RP. It was demonstrated that RIP-1 inhibited the protein unfolding activity of the 19S RP in vitro and blocked proteasome-mediated turnover of the cell cycle protein p27 in HeLa cells. In this report, we describe experiments designed to identify the molecular target of RIP-1.

RIP-1 was identified originally in a screen that registered binding of the peptoid to the intact 26S proteasome, which contains at least 34 different polypeptides.[13] To identify the RIP-1 receptor, we used a chemical cross-linking reaction that involves the oxidation of a dihydroxyphenylalanine (DOPA) moiety contained in the molecule of interest to an ortho-quinone intermediate that can then cross-link to nearby nucleophilic residues on the receptor protein (Figure 2A).[14,15] Cross-linking is observed only if the DOPA-containing molecule and the receptor are in close proximity.[16]

Biotin-DOPA-RIP-1 (BD-RIP-1; Figure 1) was synthesized (see Supporting Information) and incubated with immunopurified *Saccharomyces cerevisiae* 26S proteasome.[8] After addition of sodium periodate ($NaIO_4$) and a brief incubation, the reaction was quenched and cross-linked products were detected by two-dimensional gel electrophoresis and blotting with NeutrAvidin-Horse Radish Peroxidase (NA-HRP). As shown in Figure 2B (left side), a biotin-containing product with an apparent mass of approximately 50 kDa and an isoelectric point of approximately 5.5 was produced, along with multiple products that appeared as a smear with an apparent molecular mass of 20−25 kDa. We have previously identified all of the yeast proteasome components by two-dimensional gel

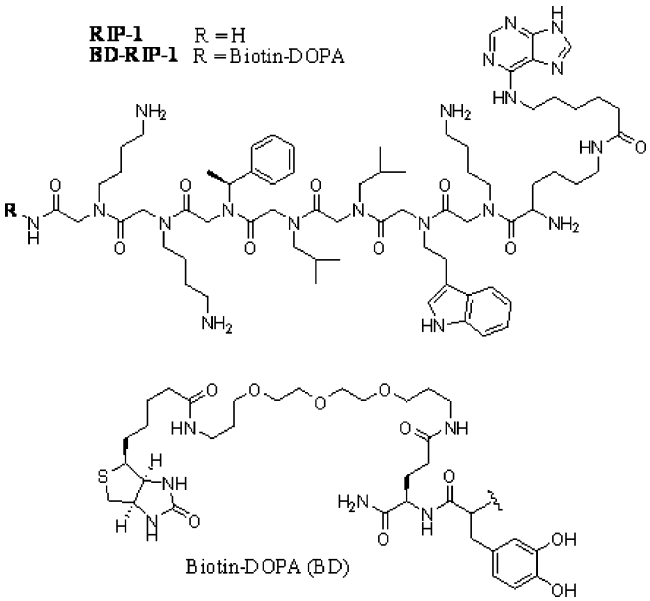

Figure 1. Chemical structures of RIP-1 and biotinylated DOPA-conjugated RIP-1, BD-RIP-1.

electrophoresis and mass spectrometry,[17] allowing us to identify the ≈50 kDa product as a cross-link between BD-RIP-1 and Sug2/Rpt4, one of the six ATPases in the proteasome. This assignment was confirmed by stripping the blot and reprobing with a highly specific antibody raised against Sug2/Rpt4. The spot on this Western blot coincides exactly with that of the ≈50 kDa biotinylated product (Figure 2B, right side), suggesting that Sug2/Rpt4 is the direct target of RIP-1.

To confirm this assignment, we performed a cross-linking experiment using recombinant Rpt proteins. Sug1/Rpt6 cannot be the ATPase cross-linked to RIP-1 because its isoelectric point (9.09) is quite different than those of the other five Rpt proteins.[18] Therefore, we employed crude cell lysates prepared from *E. coli* strains that express His6-tagged Rpts1−5 (Supporting Information Figure S2). Unfortunately, His6-Rpt3 was not soluble under any of the conditions explored, so the experiment was conducted with the remaining four ATPases. The periodate-triggered cross-linking reactions were carried out using the same protocol employed for the experiment with the full proteasome, and then quenched by adding 20 mM β-mercaptoethanol and 8 M urea. The His6-tagged Rpt protein was then precipitated using Ni-NTA resin. This denaturing protocol detects only covalently cross-linked products. The NeutrAvidin-HRP blot showed a clear cross-linked product from a lysate containing Sug2/Rpt4, while no cross-linked products were observed in the others (Figure 2C). We conclude from these results that Sug2/Rpt4 is probably the only molecular target of RIP-1 in the 19S RP. Furthermore, this result argues that the RIP-1-Rpt4

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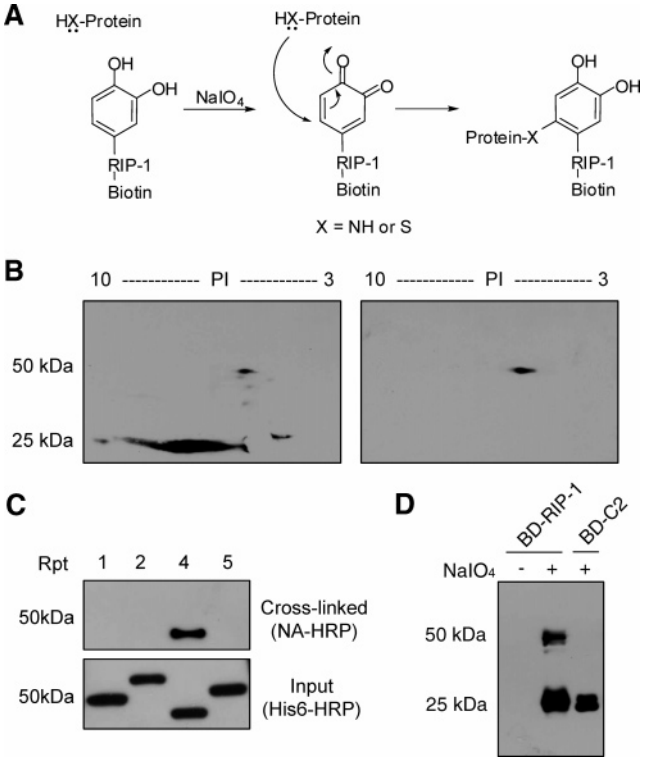

Figure 2. Chemical cross-linking identifies Sug2/Rpt4 as the major molecular target of RIP-1. (A) Proposed mechanism for oxidative chemical cross-linking reaction of DOPA-containing RIP-1 and the proteasome. (B) Left side: Periodate-triggered cross-linking of BD-RIP-1 (1 μM), followed by two-dimensional electrophoresis and Western blotting with NeutrAvidin-HRP (NA-HRP) provided a major cross-linked product with a molecular weight of approximately 50 kDa and an isoelectric point of about 5.5. Right side: Same as left side, except the blot was stripped and reprobed with anti-Sug2/Rpt4 antibody. (C) Cross-linking of BD-RIP-1 and recombinant His6-Rpts. Cross-linking reaction of BD-RIP-1 was carried out in *E. coli* lysates that express each His6-tagged Rpt protein. Each mixture was then incubated with Ni-NTA resin under denaturing conditions, and the bound protein was analyzed by Western blot with NA-HRP (upper panel). Input blotted with anti-His6 antibody showed equivalent amounts of protein were loaded (lower panel). (D) A NA-HRP blot showing the products resulting from sodium periodate treatment of the 26S proteasome and BD-RIP-1 or BD-C2.

interaction is quite specific. The six proteasomal ATPases all contain a highly conserved 200 residue AAA domain and are approximately 40% identical to one another in their sequence,[18] yet no detectable coupling between RIP-1 and the other ATPases tested was observed. However, we cannot absolutely rule out the possibility that the absence of cross-linked product is due to the lack of a nucleophile near the binding site of RIP-1 on the other Rpt proteins.

The smeared bands around 25kDa must arise from cross-linking of the RIP-1 derivative with various 20S subunits, which have similar masses. This was unexpected since RIP-1 has no effect on 20S-mediated peptidolysis (H.-S.L. et al., unpublished data) and led us to suspect that this cross-linking might simply reflect the entry of the RIP-1 derivative into the internal cavity of the 20S CP, followed by oxidation and cross-linking to 20S CP proteins in this confined space. If true, almost any biotin−DOPA−peptoid derivative should show similar cross-linked products but should not couple to the Sug2/Rpt4 protein. To test this, a DOPA−biotin derivative of a control peptoid that was not selected to bind to the proteasome (C2; see Supporting Information) was synthesized and carried through the same experiment. As shown in Figure 2D, this nonspecific peptoid indeed cross-linked to 20S components but not the Sug2/Rpt4, consistent with the idea that the 20S protein-containing products indeed do not represent specific binding of the RIP-1 peptoid to these proteins.

To validate that BD-RIP-1 retains the activity of the parent compound, RIP-1, we examined its ability to block the 19S RP-mediated unfolding of a transactivator protein, using an assay reported previously.[13] The activities of BD-RIP-1 and RIP-1 were similar, indicating that DOPA and biotin conjugation did not affect RIP-1's activity (data not shown).

In conclusion, we have presented data that identify the molecular target of the RIP-1 peptoid, the first reported chemical modulator of the 19S RP. The results also suggest that the periodate-triggered cross-linking methodology employed in this study provides a potentially powerful tool for target identification of small molecules isolated from assays that do not clearly demand the compound of interest targets a specific protein. These could include binding assays where a large, multi-protein is the target, as was the case for the protocol by which RIP-1 was identified,[13] or phenotypic assays that are used commonly in chemical genetics. RIP-1 or more potent derivatives may prove to be useful tools to probe the detailed mechanism of action of this essential ATPase in a variety of proteolytic and non-proteolytic events. These studies are underway.

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Supporting Information Available: Detailed experimental procedures and supplementary figures are available. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Baumeister, W.; Walz, J.; Zuhl, F.; Seemuller, E. *Cell* **1998**, *92*, 367−380.
(2) Goldberg, A. *Nature* **2003**, *426*, 895−899.
(3) Coux, O.; Tanaka, K.; Goldberg, A. L. *Annu. Rev. Biochem.* **1996**, *65*, 801−847.
(4) DeMartino, G.; Slaughter, C. A. *J. Biol. Chem.* **1999**, *274*, 22123−22126.
(5) Kisselev, A. F.; Goldberg, A. *Chem. Biol.* **2001**, *8*, 739−758.
(6) Adams, J. *Cancer Cell* **2004**, *5*, 417−421.
(7) Russell, S. J.; Reed, S. H.; Huang, W.; Friedberg, E. C.; Johnston, S. A. *Mol. Cell* **1999**, *5*, 687−696.
(8) Ferdous, A.; Gonzalez, F.; Sun, L.; Kodadek, T.; Johnston, S. A. *Mol. Cell* **2001**, *7*, 981−991.
(9) Gonzalez, F.; Delahodde, A.; Kodadek, T.; Johnston, S. A. *Science* **2002**, *296*, 548−550.
(10) Ezhkova, E.; Tansey, W. P. *Mol. Cell* **2004**, *13*, 435−442.
(11) Collins, G. A.; Tansey, W. P. *Curr. Opin. Genet. Dev.* **2006**, *16*, 197−202.
(12) Ferdous, A.; Sikder, D.; Gillette, T. G.; Nalley, K.; Kodadek, T.; Johnston, S. A. *Genes Dev.* **2007**, *21*, 112−123.
(13) Lim, H. S.; Archer, C. T.; Kodadek, T. *J. Am. Chem. Soc.* **2007**, *129*, 7750−7751.
(14) Burdine, L.; Gillette, T. G.; Lin, H.-J.; Kodadek, T. *J. Am. Chem. Soc.* **2004**, *126*, 11442−11443.
(15) Archer, C.; Burdine, L.; Kodadek, T. *Mol. BioSyst.* **2005**, *1*, 366−372.
(16) Liu, B.; Burdine, L.; Kodadek, T. *J. Am. Chem. Soc.* **2006**, *126*, 11442−11443.
(17) Denison, C.; Kodadek, T. *J. Proteome Res.* **2004**, *3*, 417−425.
(18) Glickman, M. H.; Rubin, D. M.; Fried, V. A.; Finley, D. *Mol. Cell Biol.* **1998**, *18*, 3149−3162.

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