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# Mechanism-Based Small Molecule Cross-Linkers of HECT E3 Ubiquitin Ligase–Substrate Pairs

Sungjin Park, Ioanna Ntai, Paul Thomas, Evgeniia Konishcheva, Neil L. Kelleher, and Alexander V. Statsuk*

Department of Chemistry, Center for Molecular Innovation and Drug Discovery, Proteomics Center of Excellence, Chemistry of Life Processes Institute, Northwestern University, Silverman Hall, 2145 Sheridan Road, Evanston, Illinois 60208, United States

Supporting Information

**ABSTRACT:** Here we report the discovery that bifunctional thiol- and amine-reactive electrophiles serve as mechanism-based covalent cross-linkers for HECT E3 ubiquitin ligase–substrate pairs. We demonstrate that these chemical cross-linkers covalently cross-link the catalytic Cys residue of the yeast HECT E3 ubiquitin ligase Rsp5 with the Lys of the ubiquitination site in the model substrate Sic60-GFP. This work represents the first example of a mechanism-based covalent cross-link of HECT E3–substrate pairs that converts transiently interacting HECT E3–substrate pairs into stable, covalently cross-linked protein complexes, thereby facilitating their subsequent isolation, identification, and study.

Protein ubiquitination is the ubiqutous type of posttranslational modifcation, which is controlled by E3 ubiquitin ligase enzymes that select specific protein substrates to be ubiquitinated.[1,2] Ubiquitin ligases can be divided into two groups based on their enzymatic mechanisms. RING and U-box ubiquitin ligases bind to their protein substrates and ubiquitin-conjugating E2 enzymes and stimulate the subsequent transfer of ubiquitin from the E2 enzyme onto the lysine of the protein substrate. HECT E3 ubiquitin ligases (~28 enzymes are known), on the other hand, form a covalent thioester intermediate with the C-terminus of ubiquitin through the conserved cysteine residue and then transfer ubiquitin directly onto the lysine residues of the protein substrate.[3,4] Mutations and amplifications of HECT E3 ubiquitin ligase genes are known to cause neurodegenerative diseases, hypertension disorders, and cancers.[5] It is therefore important to find downstream protein substrates of HECT E3s to develop a better understanding of the molecular mechanisms of these diseases. Current methods for identifying protein substrates of HECT E3 ubiquitin ligases include in vitro binding and ubiquitination assays[6] and immunoprecipitation of endogenous HECT E3 ubiquitin ligase–substrate pairs from cell lysates.[7]

The first two methods, although effective, do not recapitulate native physiological conditions. Immunoprecipitation assays, on the other hand, mimic native physiological conditions yet have limitations, because interactions of HECT E3s with protein substrates are transient and usually of low affinity.[8] To this end, the covalent cross-link of HECT E3–substrate pairs in native proteomes would enhance the stability of transient HECT E3–substrate complexes, thus facilitating their isolation, identification, and study. Interestingly, in vitro binding and enzymatic ubiquitination assays showed a correlation between the ability of HECT E3s to bind to their protein substrates in the absence of other components of the ubiquitin-conjugating machinery and the ability of HECT E3s to ubiquitinate the same protein substrates.[6b] These findings suggest that the mode of binding of the protein substrate to HECT E3 in the HECT E3–substrate complex may be similar to the mode of binding of the protein substrate to the HECT E3~Ub thioester complex in the enzymatic reaction. Because the lysine of the protein substrate must come into the proximity of the catalytic cysteine of the HECT E3~Ub thioester complex during the ubiquitination reaction, we hypothesize that in the HECT E3–substrate complex, the lysine of the protein ubiquitination site will also be proximal to the catalytic cysteine of HECT E3 ubiquitin ligase.

This paper tests the proposed hypothesis, and if this hypothesis is correct, then bifunctional cysteine and lysine reactive small molecule cross-linkers such as **1** could covalently cross-link the catalytic cysteine of HECT E3 ubiquitin ligases with the lysine of the ubiquitination site of protein substrates (Figure 1).

To test this hypothesis, we focused on HECT E3 ubiquitin ligase Rsp5 and its protein substrate Sic60-GFP. Rsp5 is an essential gene in *Saccharomyces cerevisiae* budding yeasts and regulates a diverse array of cellular functions such as mitotic chromatin assembly, protein trafficking, fatty acid synthesis, stability of RNA polymerase II, and export of mRNA from the

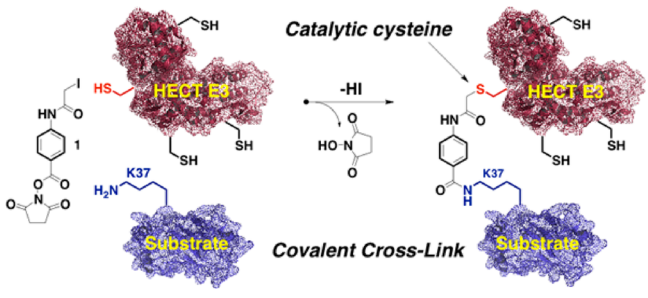


**Figure 1.** Mechanism-based small molecule cross-linkers of HECT E3 ubiquitin ligase–substrate pairs.

**Received:** September 10, 2012
**Revised:** October 5, 2012
**Published:** October 8, 2012

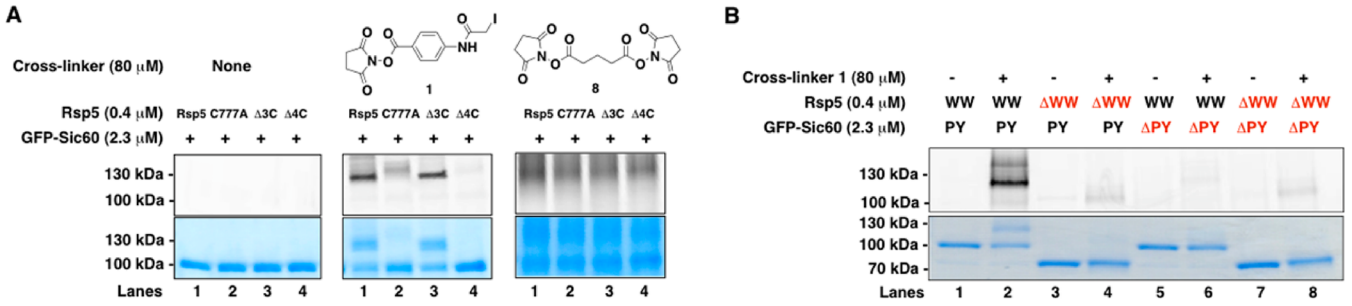


**Figure 2.** (A) Cross-linking of Rsp5, C777A, Δ3C, and Δ4C with the fluorescent Sic60-GFP protein substrate in the presence of cross-linker **1**. (B) Cross-linking of Rsp5 and Rsp5ΔWW with Sic60-GFP and Sic60-GFPΔPY protein substrates in the presence of cross-linker **1**. In both cases, the covalent cross-link was detected using in-gel fluorescence (top) and Coomassie staining (bottom).

nucleus.[9] Rsp5 contains the catalytic C-terminal HECT domain, three WW domains, and the C2 domain. WW domains contain conserved tryptophans that interact with the proline-rich motifs present in the protein substrates of Rsp5. Sic60-GFP protein is a known artificial substrate of Rsp5 and contains first 60 amino acids of protein Sic1 at its N-terminus, a proline-rich sequence of PPPY, which interacts with WW domains of Rsp5, and a GFP tag at its C-terminus.[10] The GFP tag allows convenient monitoring of Sic60-GFP ubiquitination and the covalent cross-link to Rsp5 using in-gel fluorescence imaging (Figure S1 of the Supporting Information). Indeed, treatment of the mixture of Rsp5 and Sic60-GFP with bifunctional cross-linker **1** produced a new fluorescent band with a molecular mass of ∼130 kDa that corresponds to the combined molecular mass of two proteins (Figure 2A, lane 1, cross-linker **1**, and Figure S2A of the Supporting Information). We also observed the formation of higher-molecular mass fluorescent bands (25% fluorescence intensity of the major band), because of the additional reactivity of surface cysteines in Rsp5 (Figure S2E of the Supporting Information). When Rsp5 or Sic60-GFP was treated with cross-linker **1** separately, cross-linked bands were not observed, suggesting that the presence of both Rsp5 and the Sic60-GFP substrate is necessary to produce the covalently cross-linked protein complex (Figure S2C of the Supporting Information). The cross-linking is observed even in the presence of the HeLa cell lysate (Figure S2B of the Supporting Information) without the significant decrease in the fluorescence intensity of the cross-linked band (Figure S2D of the Supporting Information).

To test if the cross-linking depends on the presence of the catalytic cysteine ($Cys^{777}$) of Rsp5, we prepared a full-length Rsp5 as well as catalytically inactive mutant Rsp5 C777A. In addition, two other mutant proteins were prepared: the Rsp5Δ3C mutant, which has all surface cysteines removed via C455A, C517S, and C721A mutations, and the catalytically inactive Rsp5Δ4C mutant, with catalytic and surface cysteines removed via C777A, C455A, C517S, and C721A mutations (Figure S3A,B of the Supporting Information). We expected that the mechanism-based thiol-to-amine cross-linkers will cross-link lysine of Sic60-GFP with the catalytic cysteine of Rsp5 and Rsp5Δ3C, but not with Rsp5 C777A or Rsp5Δ4C mutants because they lack the catalytic $Cys^{777}$ residue. As we expected, the observed cross-linking of Rsp5 and Sic60-GFP protein with cross-linker **1** was dependent on the presence of the catalytic $Cys^{777}$ residue in Rsp5 (Figure 2A). A significant decrease in the magnitude of the covalent cross-link of Rsp5 and Sic60-GFP was observed when catalytic $Cys^{777}$ was removed (Figure 2A, lanes 2 and 4). In contrast, disuccinimidyl glutarate **8**, which is frequently used to cross-link proteins via the surface lysine residues, was not able to discriminate the presence of catalytic or surface cysteine residues in Rsp5 (Figure 2B). Similar results were observed with the panel of other cross-linkers, **2**−**7**, in which the distance between thiol and amine reactive groups varied (Figure S4 of the Supporting Information).

To study the role of the protein−protein interactions in the cross-linking reaction, we prepared the Rsp5ΔWW mutant that lacks three WW domains, which are essential for the recognition of the PPPY motif of the Sic60-GFP substrate. Compared to the wild type, Rsp5ΔWW was not active in ubiquitinating Sic60-GFP, yet autoubiquitination of Rsp5ΔWW was readily detected (Figure S5 of the Supporting Information). Accordingly, treatment of Rsp5ΔWW and Sic60-GFP with cross-linker **1** did not produce a significant cross-link (Figure 2B, lanes 1−4). Similar results were obtained when $Tyr^{9}$ in the PPPY motif of Sic60-GFP was mutated to $Ala^{9}$, leading to the Sic60-GFPΔPY mutant protein, which was not polyubiquitinated by Rsp5 (Figure S6 of the Supporting Information). As expected, cross-linker **1** did not cross-link Rsp5 and Sic60-GFPΔPY (Figure 2B, lanes 5 and 6) or Rsp5ΔWW and Sic60-GFPΔPY proteins (Figure 2B, lanes 7 and 8).

To identify the cross-linked site, the covalently cross-linked complex of Rsp5 and Sic60-GFP was isolated, digested, and subjected to tandem MS/MS analysis to locate cross-linked cysteine and lysine residues on Rsp5 and Sic60-GFP, respectively (Figure S7 of the Supporting Information). We found that only the catalytic $Cys^{777}$ residue of Rsp5 was cross-linked to $Lys^{37}$ of Sic60-GFP (Figure S7B of the Supporting Information); another $Cys^{721}$ residue present in Rsp5 was alkylated with the cross-linker **1**, but not cross-linked. To identify ubiquitination sites of Sic60-GFP, monoubiquitinated Sic60-GFP was isolated, digested with trypsin, and analyzed by tandem MS. Interestingly, $Lys^{37}$ of Rsp5 was one of the four lysines that were ubiquitinated in the enzymatic reaction in addition to $Lys^{33}$, $Lys^{51}$, and $Lys^{54}$ (Figure S7D of the Supporting Information). The discovery that the same $Lys^{37}$ residue of Sic60-GFP is cross-linked to the catalytic $Cys^{777}$ residue of Rsp5 and ubiquitinated in the enzymatic reaction suggests that in both cases $Lys^{37}$ of Sic60-GFP comes into the proximity fo the catalytic $Cys^{777}$ residue of Rsp5. This observation is striking, because in contrast to the enzymatic reaction that includes the Rsp5∼Ub intermediate, in our system Rsp5 does not carry a covalently attached ubiquitin, yet the catalytic cysteine of Rsp5 is still proximal to the ubiquitination site of GFP-Sic60.


dx.doi.org/10.1021/bi301231h | *Biochemistry* 2012, 51, 8327−8329

In summary, we validated our initial hypothesis and showed that within the context of the investigated system, the reported covalent cross-linkers of Rsp5 and the model protein substrate cross-link the catalytic cysteine of Rsp5 and the ubiquitination site of GFP-Sic60. A further increase in the efficiency of the HECT E3−substrate cross-linkers can be achieved by using suitable directing groups that deliver thiol and amine reactive moieties to the active site of HECT E3s.[11] Our findings set the foundation for the further development of covalent cross-linkers of endogenous HECT E3−substrate pairs and for the study of HECT E3−substrate interactions in vitro.

## ■ ASSOCIATED CONTENT

### Supporting Information

Protocols for protein preparation, cross-linking assays, biochemical assays, and MS/MS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*E-mail: a-statsuk@northwestern.edu. Telephone: (847) 467-1875.

### Author Contributions

S.P., I.N., P.T., E.K. performed the experimental work. S.P., I.N., P.T., N.L.K., and A.V.S. analyzed the experimental work. A.V.S. wrote the manuscript with contributions of all authors.

### Funding

Funding from Northwestern University is greatly acknoweldged. A.V.S. is a Pew Scholar in the Biomedical Sciences, supported by the Pew Charitable Trusts.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank Daniel Kraut, Linda Hicke, and Andreas Matouschek for providing plasmids and protein expression protocols for Rsp5 and Sic60-GFP.

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