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科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Chemical Biology Studies of Ubiquitin Transfer with Synthetic Proteins

研究課題名（英文）Chemical Biology Studies of Ubiquitin Transfer with Synthetic Proteins

研究代表者
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研究成果の概要（和文）：我々は、serine/threonine ligation、α-ketoacid-hydroxylamine (KAHA) ligation、およびnative chemical ligationを用いた4セグメント3ライゲーションという収束型戦略によりUbc9を合成した。
細胞溶解液中に我々の合成したE2-SUMOプローブを利用し、E3リガーゼの候補を補足を試みた。その結果、CRY1の修飾を担当する複数のE3リガーゼ候補を特定することに成功した。

研究成果の学術的意義や社会的意義
We sought to form stable, semisynthetic Ubc9-SUMO conjugates, as these are known to preferentially interact with E3 ligases. At the current state of development, this is challenging to achieve by recombinant methods including genetic code expansion.

研究成果の概要（英文）：We prepared Ubc9 by a convergent four-segment, three-ligation strategy using serine/threonine ligation, α-ketoacid-hydroxylamine (KAHA) ligation, and native chemical ligation. This flexible, four-segment strategy was used to prepare wild-type Ubc9 as well as variants bearing combinations of side chain modifications.
The synthetic Ubc9 variants were evaluated in an SUMOylation assay using RanGAP1 as the substrate and recombinant E1 activating enzyme. To validate our key hypothesis that appropriately placed diazirines can selectively cross-link an E3 ligase bound to our synthetic Ubc9 probes, we selected RanBP2 as a test case. We attempted to trap RanBP2 using Sp100 as a substrate protein. The identity of ternary complex SUMO1-Ubc9-RanBP2 was confirmed by tandem mass spectrometry and isolated.
By employing our synthetic E2-SUMO probes in cell lysates for trapping of candidates E3 ligases. As a result, we could identify several candidate E3 ligases responsible for modifications of CRY1.

研究分野：有機合成化学

キーワード：タンパク質化学　有機合成化学　プロテオミクス解析　ユビキチン化　ケミカルバイオロジー

１．研究開始当初の背景

The biological functions of almost all organisms on Earth are adapted to the 24-hour solar cycle, regulated by a molecular clock called the circadian clock (CC). Dysregulation of the CC is linked with numerous health complications including sleep disorders, metabolic diseases, and cancer. The key molecules (clock proteins) involved have been identified, an achievement recognized by the 2017 Nobel Prize in Medicine. The cellular regulation of the clock proteins – PER, BMAL1, CLOCK, and CRY – by cycles of transcription, translation, cellular localization, and degradation, however, is emerging as a frontier both in the understanding of the circadian rhythm (CR) and the development of treatments for disorders influenced by the CR.

One of the most important factors in the homeostasis of proteins – including clock proteins – is the covalent attachment of small protein modifiers such as ubiquitin (Ub) and SUMO. The attachment of Ub chains to lysine residues is a multifaceted but widely studied process that is one of the determining factors in proteasome-mediated degradation and protein turnover. The function of SUMOylation – the attachment of various isoforms of another small protein – is complex, but is thought to be involved in protein localization, stability, regulation of protein-protein interactions and PTMs. Several clock proteins are reversibly SUMOylated, which is thought to be crucial for proper functioning of the biological clock. For example, modification of PER2 with different SUMO isoforms can have distinct effects on PER2: conjugation with SUMO2 leads to degradation of PER2 whereas SUMO1 conjugation enhances phosphorylation of PER2 by Casein Kinase 1, resulting in increased translocation. BMAL1 and CLOCK are also known to be SUMOylated and we have preliminary evidence for SUMOylation of CRY1. However, in most cases understanding of the extent, role, SUMO-isoform selectivity, and associated proteins is lacking.

２．研究の目的

The goal of this research proposal is to address the question: “How does SUMOylation influence and regulate the circadian rhythm?”. We are not the first to ask this question, but our team will bring new technologies and ideas to this challenging problem. We will employ a chemical biology approach to control, detect, and modulate SUMOylation pathways in living cells. Our ability to chemically synthesize SUMO isoforms and the essential SUMO-conjugating enzyme Ubc9 will allow us to introduce a wide array of functionalities into these proteins.

３．研究の方法

Established tools including RNA-Seq, antibody-based imaging, and proteomics can provide detailed information about the presence and expression level of specific RNAs and proteins and are powerful tools for elucidating the molecules involved in the CR. These approaches, however, provide little information on the presence or role of modified forms, such as SUMOylated proteins. In order to better understand the role of SUMOylation of clock proteins, we will employ the latest developments in chemical biology to 1) detect SUMOylated clock proteins, 2) manipulate the SUMOylation state of clock proteins and its effect on the CR, and 3) identify the E3 ligases responsible for SUMOylation of clock proteins. Key methodologies include chemical synthesis of modified SUMO E2 conjugating enzymes that will serve as photoaffinity traps for unknown interaction partners, including E3 ligases.

４．研究成果

(1) Chemical Synthesis of Ubc9 and Ubc9 Variants

We prepared Ubc9 by a convergent four-segment, three-ligation strategy using serine/threonine ligation (STL), α-ketoacid–hydroxylamine (KAHA) ligation, and native chemical ligation (NCL). We selected Gly34–Thr35, Lys74–Cys75, and Ile107–Thr108 as ligation sites due to the similar size of the resulting peptides and flexibility for introducing site-specific mutations. The presence of a Lys–Cys junction near the middle of the protein provided an ideal site for NCL. Segment **S1** for the STL ligation was prepared by Fmoc-SPPS on 2-Cl trityl resin. The C-terminal acid was converted to a salicylaldehyde ester. Li and co-workers have reported that thioesters are stable under STL conditions, allowing us to use a **S2** thioester—itself prepared according to the procedure of Liu and co-workers. Segments **S1** and **S2** were assembled by STL to yield peptide S1+S2.

Segments **S3** and **S4** were assembled by KAHA ligation. For the preparation of α-ketoacid segment **S3** with an N-terminal Cys residue, we employed the previously reported protected isoleucine α-ketoacid on Rink amide resin. The N-terminal cysteine residue of **S3** was protected with a *S*-*tert*-butyl thiol and the other Cys residues were protected as *S*-acetamidomethyl-l-cysteine (Fmoc-Cys(Acm)-OH) to avoid undesired disulfide bond formation during handling and purification. Segment **S4** was prepared by standard methods

with Boc-(*S*)-5-oxaproline as the N-terminal residue. The KAHA ligation of **S3** with **S4** was carried out to give the ligated product *depsi*-peptide **S3+S4**, with a homoserine (Hse) ester at the ligation site. The *O*- to *N*-acyl shift was performed under basic condition to yield peptide **S3+S4**.
The purified STL and KAHA ligation products **S1+S2** and **S3+S4** could be employed for NCL to generate the full-length Ubc9 protein. Removal of the Acm groups afforded full length synthetic Ubc9(Thr108Hse) protein. The synthetic protein was characterized by mass spectroscopy and sequenced by LC-MS/MS after chymotrypsin digestion; tandem mass spectrometry showed 100% sequence coverage of Ubc9. The linear synthetic Ubc9(Thr108Hse) was folded by dialyzing against the folding buffer and confirmed by circular dichroism in comparison to an authentic sample.
This flexible, four-segment strategy was used to prepare wild-type Ubc9 as well as variants bearing combinations of side chain modifications, a task that—at the current state of development—is challenging to achieve by recombinant methods including genetic code expansion. We sought to form stable, semisynthetic Ubc9–SUMO conjugates, as these are known to preferentially interact with E3 ligases. We postulated that 2,3-diaminopropionic acid (Dap), which substitutes a primary amine for the cysteine thiol, could form a stable amide bond between Ubc9 and SUMO, thereby introducing minimal perturbation of the interactions with E3 ligases. However, no examples of E2 conjugating enzymes bearing this modification have been reported, and our synthetic approach allowed facile incorporation of the Cys93Dap mutation simply by employing the appropriate amino acid 2,3-diaminopropionic acid during the synthesis of Segment 3. We also sought to incorporate amino acids bearing side chains suitable for photo-cross-linking protein–protein interactions. To this end, amino acid residues containing diazirines as photoinduced cross-linkers were installed at various sites including Phe22, Tyr68, Pro69, and Pro105. Finally, to facilitate affinity purification and biochemical detection, we also installed an N-terminal biotin tag. In all cases, the syntheses of these Ubc9 variants proceeded smoothly without complications from the unnatural amino acids or diazirine moieties.

(2) Biochemical Assay of Synthetic Ubc9 and Formation of Covalent Ubc9–SUMO Conjugates
The synthetic Ubc9 variants were evaluated in an ATP-dependent SUMOylation assay using RanGAP1 as the substrate and recombinant E1 activating enzyme. The activity of synthetic Ubc9(Thr108Hse) was indistinguishable from recombinant Ubc9, and RanGAP1 was SUMOylated for all three SUMO isoforms, SUMO1, SUMO2, and SUMO3.
The biotinylated Ubc9 variants bearing the Cys93Dap mutation were evaluated for their ability to form a covalent complex with SUMO. We performed the *in vitro* conjugation reaction by incubating Ubc9(Cys93Dap, Thr108Hse, N-biotin) with SUMO1 in the presence of activating enzyme E1 and ATP. For Ubc9(Thr108Hse, N-biotin), the single SUMOylated form was observed, which is consistent with a published result. SUMO1 was attached to Ubc9(Cys93Dap, Thr108Hse, N-biotin), and its conjugation was dependent on ATP and E1.
To confirm the identity of the conjugate, we treated the protein bands corresponding to Ubc9(Cys93Dap, Thr108Hse, N-biotin)–SUMO1 with trypsin and chymotrypsin and analyzed the resulting digests by tandem mass spectrometry. A peptide remnant from the C terminus of SUMO1 was found to be conjugated to the target mutation residue Dap93 of Ubc9(Cys93Dap, Thr108Hse, N-biotin).

(3) Trapping of Model substrate
To validate our key hypothesis that appropriately placed diazirines can selectively cross-link an E3 ligase bound to our synthetic Ubc9 probes, we selected RanBP2 as a test case. RanBP2 is one of the best characterized E3 ligases and has been reported to enhance Sp100 SUMOylation with a marked preference for SUMO1 over SUMO2/3. The interaction of RanBP2 with conjugating enzyme Ubc9 has been determined by X-ray crystallography, and this structure highlighted that the hydrophobic side chain of Phe22 of Ubc9 was located in the exposed loop region and presented at the binding surface between RanBP2 and Ubc9. Accordingly, we chose Phe22 to incorporate the photoreactive Phe derivative l-(4-Tmd)-Phe (represented as F*) and prepared synthetic Ubc9 variants bearing this F22F* mutation.
To test the reactivity of the diazirine moiety incorporated into these Ubc9 variants, we attempted to trap RanBP2 using Sp100 as a substrate protein. When the Cys93Dap mutant Ubc9(F22F*, Cys93Dap, Thr108Hse, *N*-biotin) was incubated with SUMO1, Sp100, and RanBP2 in the presence of E1 and ATP at room temperature for 1 h, followed by UV irradiation, we observed clean formation of a new band that could be detected by anti-biotin (synthetic Ubc9), anti-SUMO1, and anti-GST (attached to RanBP2). The identity of ternary complex SUMO1–Ubc9(F22F*, Cys93Dap, Thr108Hse, *N*-biotin)–RanBP2 was confirmed by tandem mass spectrometry and isolated by affinity purification with streptavidin beads.

(4) Trapping of E3 ligases responsible for modifications of CRY1
By employing our synthetic E2-SUMO probes in cells and cell lysates for photoinduced trapping of candidates E3 ligases. By working with our collaborator Dr. Kuwata, we could identify several candidate

E3 ligases responsible for modifications of CRY1. Based on these hits, we will continue to work with another collaborator, Dr. Tsuyoshi Hirota, to validate these findings using siRNA knockdowns of the candidate genes.

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５．主な発表論文等

〔雑誌論文〕　計6件（うち査読付論文　5件／うち国際共著　4件／うちオープンアクセス　1件）

１．著者名 Farnung Jakob、Song Haewon、Bode Jeffrey W.	４．巻 -
２．論文標題 Chemical Protein Synthesis by Chemoselective a-Ketoacid-Hydroxylamine (KAHA) Ligations with 5-Oxaproline	５．発行年 2021年
３．雑誌名 Peptide Conjugation. Methods in Molecular Biology	６．最初と最後の頁 151～162
掲載論文のDOI（デジタルオブジェクト識別子） 10.1007/978-1-0716-1617-8_14	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Zhang Yinfeng、Hirota Tsuyoshi、Kuwata Keiko、Oishi Shunsuke、Gramani Subramanian G.、Bode Jeffrey W.	４．巻 141
２．論文標題 Chemical Synthesis of Atomically Tailored SUMO E2 Conjugating Enzymes for the Formation of Covalently Linked SUMO-E2-E3 Ligase Ternary Complexes	５．発行年 2019年
３．雑誌名 Journal of the American Chemical Society	６．最初と最後の頁 14742～14751
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/jacs.9b06820	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Rohrbacher Florian、Zwicky Andre、Bode Jeffrey. W.	４．巻 -
２．論文標題 Facile Synthesis of Internal and C-Terminal Peptide α-Ketoamides with Fmoc-Solid Phase Peptide Synthesis	５．発行年 2018年
３．雑誌名 Helvetica Chimica Acta	６．最初と最後の頁 -
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オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Bode Jeffrey W.	４．巻 50
２．論文標題 Chemical Protein Synthesis with the α-Ketoacid-Hydroxylamine Ligation	５．発行年 2017年
３．雑誌名 Accounts of Chemical Research	６．最初と最後の頁 2104～2115
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acs.accounts.7b00277	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

1．著者名 Harmand Thibault J.、Pattabiraman Vijaya R.、Bode Jeffrey W.	4．巻 56
2．論文標題 Chemical Synthesis of the Highly Hydrophobic Antiviral Membrane-Associated Protein IFITM3 and Modified Variants	5．発行年 2017年
3．雑誌名 Angewandte Chemie International Edition	6．最初と最後の頁 12639～12643
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/anie.201707554	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

1．著者名 Rohrbacher Florian、Zwicky Andre、Bode Jeffrey W.	4．巻 8
2．論文標題 Chemical synthesis of a homoserine-mutant of the antibacterial, head-to-tail cyclized protein AS-48 by α-ketoacid-hydroxylamine (KAHA) ligation	5．発行年 2017年
3．雑誌名 Chemical Science	6．最初と最後の頁 4051～4055
掲載論文のDOI（デジタルオブジェクト識別子） 10.1039/C7SC00789B	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
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7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関