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Ubiquitination of an artificial RING finger without a substrate and a tag

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Alpha-helical region substitution was applied to the SIAH1 and EL5 RING fingers. The Williams–Beuren syndrome transcription factor (WSTF) PHD_SIAH1 and WSTF PHD_EL5 RING fingers were created as the artificial ubiquitin-ligating enzyme (E3). These fingers possess E3 activities of mono-ubiquitination and poly-ubiquitination, respectively, with ubiquitin-conjugating enzyme (E2)-binding capabilities. Artificial E3s bind two zinc atoms and adopt a zinc-dependent ordered structure and ubiquitinate upon themselves without a substrate and a tag. Ubiquitination experiments using biotinylated ubiquitin showed that the WSTF PHD_EL5 RING finger is poly-ubiquitinated via residue Lys^{63} of ubiquitin. Substitution of alpha-helical region might be applicable to various RING fingers with mono-ubiquitination or poly-ubiquitination.

Supporting information may be found in the online version of this article.

Keywords: RING finger; PHD finger; ubiquitination; E3; artificial E3; zinc-binding protein

Introduction

The RING finger is a zinc-binding motif with residues Cys and/or His [1–3]. Protein ubiquitination is undertaken through an enzymatic cascade comprising ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes [4,5]. The ubiquitination system is associated with cancers and Parkinson's disease (PD) [6]. E3s can be utilized as cancer targets/biomarkers for diagnosis and prognosis [7]. Seven in absentia homolog-1 (SIAH1) of E3 mediates mono-ubiquitination of the substrate α-synuclein related to PD [8,9].

Most RING fingers function as E3 and transfer the activated ubiquitin from E2 to the ε-amino groups of the substrate Lys [10–12]. If the substrate is absent, RING fingers such as EL5 and SIAH1 cannot secure the destination for transferring ubiquitin. By adding a tag such as maltose-binding protein (MBP), MBP-EL5 RING finger fusion constructs exhibit E3 activity and can transfer ubiquitin to the MBP moiety [13].

To evaluate if a RING finger exhibits E3 activity in the ubiquitination reaction, a substrate or a tag (e.g., MBP) is required. If the substrate is not identified, design of the fusion constructs of the RING finger with a tag is required. In a small construct of the MBP-EL5 RING finger fusion, the MBP moiety prevents the development of the poly-ubiquitination reaction because of steric hindrance [13]. In the ubiquitination assay using a tag, a construct for avoiding steric hindrance to the development of the poly-ubiquitin chain must be designed.

'Alpha-helical region substitution' has been reported for the design of artificial RING fingers [14]. E3 RING fingers have a groove for the E2-binding site including the essential α-helical region which contributes to the specific E2–E3 binding capabilities [15]. If the helical region of the EL5 RING finger is inserted into the amino-acid sequence between the sixth and seventh zinc ligands of the Williams–Beuren syndrome transcription factor (WSTF) PHD finger, the artificial RING finger (WSTF PHD_EL5 RING finger) cooperates with UbcH5. It is then poly-ubiquitinated upon itself without a substrate and a tag (Figure 1).

In this study, to extend the application of the strategy, Alpha-helical region substitution was newly applied to the SIAH1 RING finger that catalyzes the mono-ubiquitination of α-synuclein rather than the poly-ubiquitination. The present study provides the first demonstration for the creation of the artificial E3 with mono-ubiquitination and specific E2 binding capabilities. Furthermore, to examine the property for poly-ubiquitin chains of the artificial RING finger, the type specificity of Lys linkages on the artificial WSTF PHD_EL5 RING finger was assessed by ubiquitination experiments using biotinylated ubiquitin.

Materials and Methods

Synthesis of Artificial WSTF PHD_RING Fingers

Artificial WSTF PHD_RING fingers were synthesized using a standard F-moc solid-phase strategy. Chemicals for peptide assembly, including amide resin, were obtained as SynProPep products from Shimadzu Corp. (Kyoto, Japan). After cleavage with trifluoroacetic acid, the peptides were purified by a reversed-phase high-performance liquid chromatography system using a Shim-pack C18 column (Shimadzu). Peptide purity was >98%, and its molecular mass was assessed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Shimadzu AXIMA-TOF^2. Obtained peptides were dissolved in

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Abbreviations used: *E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-ligating enzyme; MBP, maltose-binding protein; RING, really interesting new gene; PHD, plant homeo domain; SIAH1, seven in absentia homolog-1.*

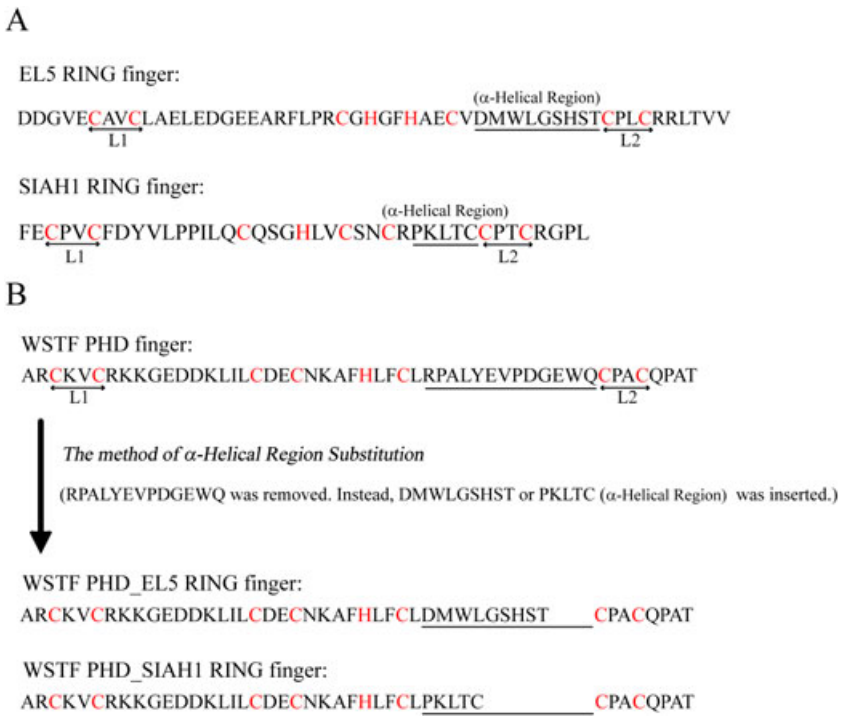

Figure 1. (A) Amino-acid sequences of the EL5 and SIAH1 RING fingers. (B) Alpha-helical region substitution for the design of an artificial RING finger. The amino-acid sequences related to substitution of the α-helical region are underlined. Zinc ligands are shown in red. L1 and L2 are the short loops corresponding to the amino-acid sequences between zinc ligands, the first and fourth pairs, respectively.

2 ml of 8 M guanidine-HCl and dialyzed against solution A [20 mM Tris-HCl (pH 6.8), 50 mM NaCl, 1 mM dithiothreitol, and 50 μM $ZnCl_2$] overnight at 4 °C.

Stoichiometry of Released Zinc Ions

Williams–Beuren syndrome transcription factor PHD_RING finger's concentration was spectrophotometrically determined by the Bradford method against bovine serum albumin. By chemical modification of Cys residues using *p*-(hydroxymercuri)benzoic acid (PHMB) and 4-(2-pyridylazo)resorcinol (PAR), the concentration of the zinc atoms released at room temperature was estimated using the equation

$$A = \varepsilon cl,$$

where molar absorptivity (ε) is 6.6×10^4 M^{-1} cm^{-1}, cell length (l) is 1.0 cm, the absorbance value (A) is recorded at the saturation plateau at 500 nm, and c represents molecular concentration. Zinc : protein stoichiometry was estimated from the ratio between the amount of the released zinc atoms and the WSTF PHD_RING finger [14,16,17].

Circular Dichroism Spectroscopy

Circular dichroism (CD) data were recorded on a J-805 spectropolarimeter (JASCO Corp.,Tokyo, Japan) using a quartz cell (path length, 1 mm) after calibration using d-camphor-10-sulfonate. The sample (25 μM) was dissolved in solution A. Spectra at 20 °C were obtained at 195–250 nm using a scanning speed of 50 nm/min, a response time of 4.0 s, and a bandwidth of 1 nm. CD spectrum was the average of four scans. After subtraction of the solvent spectrum, the CD data were converted from CD signals into mean residue molar ellipticity.

In Vitro Substrate-Independent Ubiquitination

The ubiquitination reaction was carried out in 50 μl of ubiquitination buffer (20 mM Tris-HCl (pH 6.8), 5 mM Mg-ATP, 1 mM dithiothreitol, 20 U/ml inorganic pyrophosphatase (Sigma, St. Louis, MO, USA), and 50 μM $ZnCl_2$) containing 2.5 μM biotinylated ubiquitin, 0.1 μM human recombinant E1 (His_6-tagged), and 2.5 μg E2 (His_6-tagged). Randomly biotinylated ubiquitin (Ub), Lys^6/Lys^{48}-dibiotinylated ubiquitin (Lys^6/Lys^{48}-Ub), and Lys^6/Lys^{63}-dibiotinylated ubiquitin (Lys^6/Lys^{63}-Ub) were used for the development of the ubiquitin chain. Biotinylated ubiquitin, human E1, and E2 were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Eleven E2s (UbcH1, UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8, UbcH10, and UbcH13/Mms2) were assayed in the ubiquitination reaction. The reaction mixtures also included 10 μM of the WSTF PHD_RING finger as E3. The mixtures were gently mixed and incubated at 37 °C for 60 min. The reactions were quenched by the addition of non-reducing sodium dodecyl sulfate (SDS) sample buffer. The obtained samples were separated by 10–20 % SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. For detection of biotinylated ubiquitin, the membrane was reacted with streptavidin-horseradish peroxidase solution (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) and developed by enhanced chemiluminescence (ECL, GE Healthcare, BUCKS, UK) of Western blotting detection reagent, according to manufacturer's instructions. The emitted signals were detected on a Luminescent Image Analyzer (LAS-3000, Fujifilm, Tokyo, Japan).

Results and Discussion

WSTF PHD_SIAH1 RING Finger Binds Two Zinc Atoms

Cysteine residues of the WSTF PHD_SIAH1 RING finger were chemically modified by using PHMB. The amount of zinc atoms

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released was quantified using a metallochromic indicator PAR. WSTF PHD_SIAH1 RING finger's concentration was 2.0 μM. The absorbance value (*A*) recorded at the saturation plateau was 0.29, indicating that 4.4 μM of zinc atoms was released. The molar ratio ([Zn]/[protein]) calculated for the WSTF PHD_SIAH1 RING finger was 2.20. Hence, the WSTF PHD_SIAH1 RING finger binds two zinc atoms.

Zinc-dependent Folding of the WSTF PHD_SIAH1 RING Finger

The CD spectra were obtained to estimate the folding property of the WSTF PHD_SIAH1 RING finger (Figure 2). The CD spectrum in the presence of Zn^{2+} showed double-negative minima occurring at approximately 224 nm (n–π* transitions) and 207 nm (π–π* transitions) characteristic of a typical helical structure [18,19]. Addition of iminodiacetic acid as a chelating agent resulted in unfolding of the structure, indicating that two zinc atoms are indispensable for proper folding of the WSTF PHD_SIAH1 RING finger. This finding suggested that it adopts a zinc-dependent conformation with C_4HC_3-type zinc coordination.

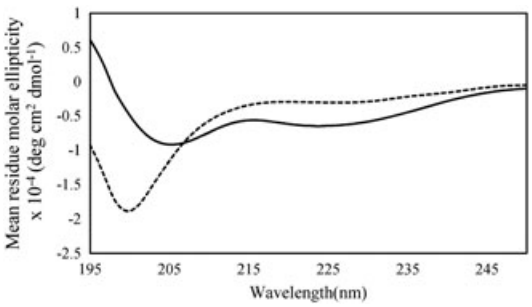

Figure 2. Circular dichroism spectra of WSTF PHD_SIAH1 RING finger. CD spectra were recorded in 20 mM Tris-HCl (pH 6.8), 50 mM NaCl, 1 mM dithiothreitol, 50 μM $ZnCl_2$, and 25 μM WSTF PHD_SIAH1 RING finger at 20 °C in the absence (solid line) or presence (dotted line) of 1 mM iminodiacetic acid.

E3 Activity and E2 Specificity of the WSTF PHD_SIAH1 RING Finger

To evaluate E3 activity and E2 specificity of the WSTF PHD_SIAH1 RING finger, ubiquitination assays were carried out with 11 E2s (UbcH1, UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8, UbcH10, and UbcH13/Mms2) in substrate-independent reactions. Formation of ubiquitin thioester-linked E2 conjugates could occur in the reaction system even in the absence of E3 [20,21]. Addition of the WSTF PHD_SIAH1 RING finger led to the accumulation of ubiquitin–E3 conjugates (Figure 3). The WSTF PHD_SIAH1 RING finger was mono-ubiquitinated in the presence of UbcH5a, UbcH5b, UbcH5c, UbcH6, or UbcH8, but not UbcH1, UbcH2, UbcH3, UbcH7, UbcH10, or UbcH13/Mms2. Poly-ubiquitination of the WSTF PHD_SIAH1 RING finger could not be observed if 11 E2s were incubated together. Therefore, the artificial WSTF PHD_SIAH1 RING finger possesses E3 activities of mono-ubiquitination with capabilities of specific E2-binding.

SIAH1 functions as E3 via its N-terminal RING finger and mono-ubiquitinates α-synuclein [22]. It belongs to the E3 group, which cooperates with UbcH5 or UbcH8 [8,9]. The SIAH1 RING finger could not be ubiquitinated upon itself in the ubiquitination reactions in the absence of the substrate (Supplementary Figure S1) because of the lack of Lys ε-amino groups in the amino-acid sequence except for the helical region (the major scaffold for the formation of the E2-binding sites) [15,23]. The helical region of the WSTF PHD_SIAH1 RING finger is derived from that of the SIAH1 RING finger. Thus, the WSTF PHD_SIAH1 RING finger possesses very similar E3 activities to those of the SIAH1 RING finger. In the absence of the substrate and tag (e.g., MBP), the WSTF PHD_SIAH1 RING finger promotes mono-ubiquitination upon itself. The EL5 RING finger also is not poly-ubiquitinated upon itself and transfers ubiquitin to the MBP moiety in the fusion constructs of the MBP tag [13,14]. The WSTF PHD_EL5 RING finger engineered using the helical region of the EL5 RING finger is poly-ubiquitinated upon itself in the absence of the MBP moiety [14]. Thus, substitution of the helical region appears to be applicable to RING fingers with poly-ubiquitination and mono-ubiquitination. The mono-ubiquitination of SIAH1 is significant for promoting the aggregation of α-synuclein [9]. In contrast, in the development of

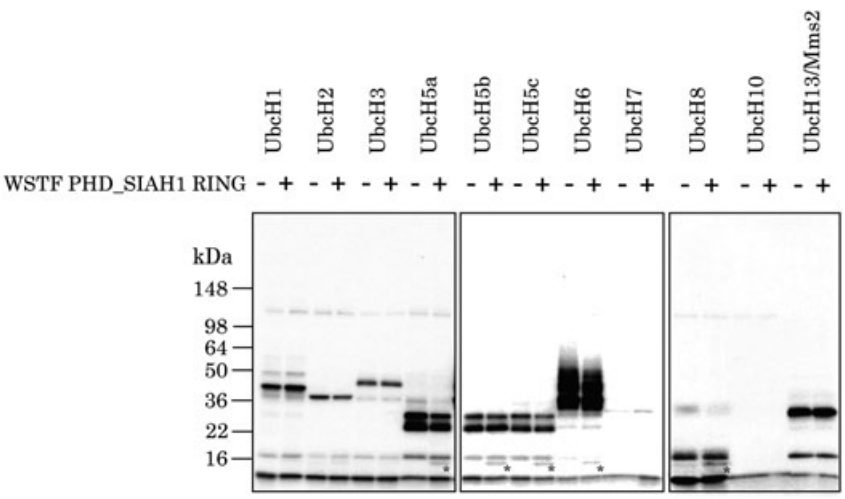

Figure 3. WSTF PHD_SIAH1 RING finger acts as an E3 ligase. The WSTF PHD_SIAH1 RING finger was added to the substrate-independent *in vitro* ubiquitination reaction containing randomly biotinylated ubiquitin (Ub), recombinant E1, and 11 E2s. Analyses were performed in the absence or presence of the WSTF PHD_SIAH1 RING finger as E3. The bands corresponding to the mono-ubiquitination products are marked with a star. The biotinylated ubiquitin on the PVDF membrane was reacted with streptavidin-horseradish peroxidase solution and developed with ECL reagent. The emitted signals were detected on a Luminescent Image Analyzer LAS-3000.

poly-ubiquitin chains, the type specificity of ubiquitin as the target signal depends upon the function such as proteasomal degradation and DNA repair, which will be examined later by ubiquitination experiments using three biotinylated ubiquitins.

The RING fingers of E3s always have the groove for binding E2, which is formed by L1, L2, and α-helical region (Figure 1) [23]. L1 and L2 are the short loops corresponding to the amino-acid sequences between zinc ligands, the first and fourth pairs, respectively. The zinc fingers of RING, PHD, ZZ, MYND, and FYVE adopt the cross-brace structure with two zinc atoms [24–27]. Their structures are very similar to one another except for the helical region. Most PHD fingers exist in the nucleus and are associated with the transcriptional control and have several Lys residues in their amino-acid sequences [24,28]. Accordingly, the PHD finger was selected as the basic construct for creating the artificial RING finger. In the helical region, the RING finger adopts the ordered helical structure, whereas the PHD finger possesses the long loop including the short helical structure. The length of the helical region is irrelevant to the selection of the appropriate basic construct from among the PHD fingers. The WSTF PHD finger is not able to ubiquitinate upon itself [14], and then the substitution of the α-helical region enables it to possess the ubiquitination capability for functioning as E3. The helical region of the E3 RING fingers is associated with specific E2–E3 binding capabilities [15]. Mutation of the residues in the catalytic core of Cdc34 as E2 causes alteration from poly-ubiquitination to mono-ubiquitination [29]. The structural diversity of the E2–E3 binding combinations probably regulates the mechanisms of poly-ubiquitination and mono-ubiquitination [30]. Taken together, these data suggested that the helical region of the RING fingers and the catalytic core of E2s contribute to the selection mechanisms of poly-ubiquitination and mono-ubiquitination.

Ubiquitin Chain-type Specificity of the WSTF PHD_EL5 RING Finger

To obtain information on the type specificity of the poly-ubiquitin chain of the WSTF PHD_EL5 RING finger, Ub, Lys^6/Lys^{48}-Ub, and Lys^6/Lys^{63}-Ub were used in the ubiquitination reactions. Ubiquitin has seven Lys residues, of which the Lys^{63}-linked poly-ubiquitin chain is associated with the target signal for the 26S proteasome, DNA repair, signal transduction, and endocytosis [31]. The Lys^{48}-linked chain is also related to the target for the proteasomal degradation [7]. The randomly biotinylated ubiquitin does not have the specified Lys residues with the modification of biotin and therefore allows seven Lys residues to develop the poly-ubiquitin chain. In the Lys^6/Lys^{48}-dibiotinylated and Lys^6/Lys^{63}-dibiotinylated ubiquitin, the modification leads to non-receptive Lys residues for the ubiquitin conjugation system [32]. Ubiquitination assays of the WSTF PHD_EL5 RING finger were performed with three biotinylated ubiquitins, and it was poly-ubiquitinated with Ub or Lys^6/Lys^{48}-Ub but not Lys^6/Lys^{63}-Ub (Figure 4). Hence, the WSTF PHD_EL5 RING finger promotes Lys^{63}-linkages for poly-ubiquitin chains such as the proteasomal targeting signal. In Lys^6/Lys^{48}-Ub, the mono-ubiquitination and poly-ubiquitination were not developed, and thus the products corresponding to the molecular weight around 148, 50, and 16 kDa could not be observed. As for Lys^6/Lys^{48}-Ub, the products of the higher molecular weight around 148 kDa were observed rather than those around 50 and 16 kDa, and hence, the poly-ubiquitination was preferentially on the WSTF PHD_EL5 RING finger. In the case of Ub, the various products around 148, 50, and 16 kDa were observed. It is suggested that the use of

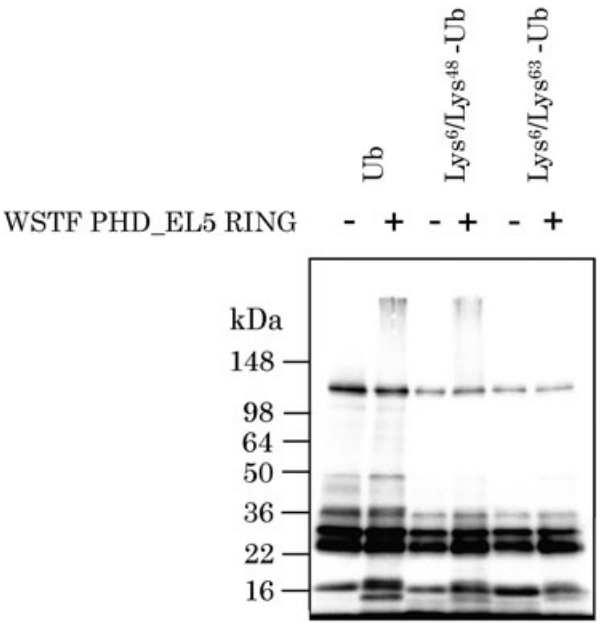

Figure 4. Poly-ubiquitination of the WSTF PHD_EL5 RING finger via residue Lys^{63} of ubiquitin. Assays were performed with Ub, Lys^6/Lys^{48}-Ub, and Lys^6/Lys^{63}-Ub. E1 and E2 (UbcH5a) were also incubated together in the absence or presence of the WSTF PHD_EL5 RING finger as E3.

Lys^6/Lys^{48}-Ub facilitates the poly-ubiquitination on the artificial E3, as compared with Ub. The compatibility between the Lys residues on the substrate and the residues around the catalytic region of E2 is considered to contribute to the specificity control for the Lys selection [30]. Selection of the Lys residues on the WSTF PHD_EL5 RING finger probably depends upon the residues around the catalytic region of E2.

On the other hand, E3s such as Hdm2 [7] and UHRF1 [33] are molecular markers for the diagnosis and prognosis in the ubiquitination system. E2s are also associated with tumor progression and are overexpressed in human cancers [34]. For the monitoring of tumor progression, the artificial WSTF PHD_RING finger can be utilized for the detection of the expression and activity of E2s without the targeting substrates. The design of the fusion constructs for transferring ubiquitin is not needed in the ubiquitination assay. The present method might be a useful tool for design of the artificial E3 as markers.

In conclusion, Alpha-helical region substitution was applied to the SIAH1 and EL5 RING fingers. The WSTF PHD_SIAH1 and WSTF PHD_EL5 RING fingers possess E3 activities of mono-ubiquitination and poly-ubiquitination, respectively, with ubiquitin-conjugating enzyme (E2)-binding capabilities. Development of poly-ubiquitination of the artificial WSTF PHD_EL5 RING finger was achieved via residue Lys^{63} of ubiquitin. The method of Alpha-helical region substitution might be applicable to various RING fingers.

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