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Differential polyubiquitin recognition by tandem ubiquitin binding domains of Rabex-5

Donghyuk Shin [a], Sei Young Lee [a], Seungsoo Han [a], Shuo Ren [b], Soyoun Kim [b], Yoshikatsu Aikawa [c], Sangho Lee [a,*]

[a] Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] Department of Biomedical Engineering, Dongguk University, Seoul 100-715, Republic of Korea
[c] Laboratory of Molecular Neurobiology, Brain Development and Aging Research Center, Doshisha University, Kyoto 610-0394, Japan

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ABSTRACT

Linkage-specific polyubiquitination regulates many cellular processes. The N-terminal fragment of Rabex-5 (Rabex-5_{9-73}) contains tandem ubiquitin binding domains: A20_ZF and MIU. The A20_ZF-MIU of Rabex-5 is known to bind monoubiquitin but molecular details of polyubiquitin binding affinity and linkage selectivity by Rabex-5_{9-73} remain elusive. Here we report that Rabex-5_{9-73} binds linear, K63- and K48-linked tetraubiquitin (Ub_4) chains with K_d of 0.1–1 μM, determined by biolayer interferometry. Mutational analysis of qualitative and quantitative binding data reveals that MIU is more important than A20_ZF in linkage-specific polyubiquitin recognition. MIU prefers binding to linear and K63-linked Ub_4 with sub μM affinities. However, A20_ZF recognizes the three linkage-specific Ub_4 with similar affinities with K_d of 3–4 μM, unlike ZnF4 of A20. Taken together, our data suggest differential physiological roles of the two ubiquitin binding domains in Rabex-5.

1. Introduction

Ubiquitination is a major regulatory mechanism controlling a myriad of cellular processes [1,2]. Ubiquitin molecules can be covalently attached to a target molecule in single or multiple molecules per chain, referred to as monoubiquitination and polyubiquitination, respectively. Polyubiquitination can be linkage-specific, meaning that one of seven lysine residues on the ubiquitin molecule becomes covalently connected to the next ubiquitin molecule via an isopeptide bond. Among the linkage-specific polyubiquitin chains, K48-linked and K63-linked polyubiquitin chains are known to be involved in distinct biological functions: K48-linked polyubiquitin chains are associated with proteasomal degradation and K63-linked ones with regulatory cellular processes [3]. Linear ubiquitination in which ubiquitin molecules are connected by the amino-terminus to the carboxy-terminus direction has recently attracted scientific interests. Linear polyubiquitin chains have been reported to be involved in activating NF-κB signaling by NEMO, a regulatory subunit of IKK complex [4]. Structurally, linear polyubiquitin chains are very similar to K63-linked polyubiquitin although they are differentially recognized in some instances [5].

Either monoubiquitin or polyubiquitin moiety is recognized by various ubiquitin binding domain (UBD)-containing proteins or ubiquitin receptors via non-covalent interactions [6,7]. UBDs recognize linkage-specific polyubiquitin chains with different selectivity. Tandem ubiquitin-interacting motifs (UIMs) of RAP80 specifically recognize K63-linked [8] and linear polyubiquitin [9]. Ubiquitin-associated (UBA) domains of isopeptidase T recognize linear and K48-linked polyubiquitins [10]. Coiled-coil region of NEMO binds linear and K63-linked polyubiquitins [11], while full-length NEMO is recently shown to bind linear polyubiquitin preferentially [12].

Rabex-5 is a guanine exchange factor for Rab5, a small GTPase regulating intracellular trafficking [13]. Rabex-5 contains multiple functional domains: two UBDs–A20-type zinc finger (A20_ZF) and motif-interacting with ubiquitin (MIU) – at the N-terminus; catalytic core comprising helical bundle and Vps9 domains in the middle and coiled coil region at the C-terminus. A20_ZF domain possesses an E3 ubiquitin ligase activity in combination with UbcH5C [14,15]. Ras proteins have been shown to be substrates for the E3 ligase activity of A20_ZF [16,17]. Rabex-5 uses its tandem UBDs (A20_ZF-MIU) for binding EGFR [18].

Interaction between the tandem UBDs of Rabex-5 and monoubiquitin has been characterized extensively both structurally and biochemically [15,18]. Each of the tandem UBDs binds monoubiquitin with K_d of 30 μM, resulting the combined K_d of 10 μM. Although less characterized, it has been implicated that A20_ZF-MIU of Rabex-5 recognizes polyubiquitin chains. A20_ZF-MIU of Rabex-5 reportedly recognizes K48-linked Ub_{2-7}. MIU domains,

* Corresponding author. Fax: +82 31 290 7015.
E-mail address: sangholee@skku.edu (S. Lee).

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when isolated, were shown to bind K63- and K48-linked Ub_{2-7} and K11-linked Ub_2 [18,19]. However, it remains unclear whether A20_ZF alone can recognize polyubiquitin chains and how MIU contributes to polyubiquitin recognition in the context of the tandem UBDs in Rabex-5. Interestingly, ZnF4 of A20, a negative regulator of NF-κB signaling, was reported to preferably bind K63-linked Ub_3 over linear, K48-linked and K11-linked Ub_3 [20].

To elucidate the molecular interactions between the tandem UBDs of Rabex-5 and polyubiquitin chains systematically, we investigated the binding of the tandem UBDs of Rabex-5 with three types of linkage-specific polyubiquitins – linear, K63-linked and K48-linked tetraubiquitin (Ub_4) chains – both qualitatively and quantitatively.

2. Materials and methods

2.1. Protein expression and purification

Preparation of plasmids encoding bovine Rabex-5_{9-73} wild-type and its mutants and human ubiquitin as a glutathione *S*-transferase (GST) fusion protein was described before [15]. The triple and sextuple mutants of Rabex-5_{9-73} were prepared using QuikChange kit (Stratagene). The plasmid encoding human linear Ub_4 as a GST fusion protein was constructed as described elsewhere [9]. K63-linked and K48-linked Ub_4 were purchased from Boston Biochem. GST-Rabex-5_{9-73} and GST-linear Ub_4 were expressed in *Escherichia coli* strain BL21(DE3). Cells were grown at 37 ℃ until optical density (OD) at 600 nm reached 0.5 ∼ 0.6, induced with 1 mM (final concentration) isopropyl-β-d-thiogalactoside and further grown at 20 ℃ overnight. The cells were harvested by centrifugation at 4,000 rpm for 20 min. Harvested cells were lysed by sonication in buffer A (50 mM Tris–HCl pH 7.5 and 150 mM NaCl) and the cell lysates were centrifuged at 13,000 rpm for 1 h at 4 ℃. The supernatant was applied to a glutathione-Sepharose resin (GE HealthCare) and the GST was cleaved using tobacco etch virus protease. The cleaved samples were loaded to a Superdex 200 16/60 column (GE HealthCare) in the buffer A.

2.2. GST pull-down assay

1 μg of GST- Rabex-5_{9-73} and its mutants were incubated with 30 μl of glutathione-Sepharose beads (GE HealthCare) pre-equilibrated with the buffer A. After incubation for 30 min, unbounded proteins were washed out three times by the buffer A. Each of linear, K63-linked and K48-linked Ub_4 was then added. The resulting solution was incubated at 4 ℃ for 2 h with gentle rocking motion on a rotating platform. To minimize non-specific binding, buffer B (50 mM TrisHCl pH 7.5, 150 mM NaCl and 0.05% (v/v) NP-40) was used for washing out unbounded Ub_4. Samples were analyzed by SDS–PAGE followed by immunoblotting with a mouse monoclonal anti-ubiquitin (Santa Cruz) with anti-mouse IgG-HRP (Sigma) antibodies and GST-HRP antibodies (GE Healthcare). For the negative control, same amount of GST protein was used in the pull-down assay.

2.3. Biolayer interferometry

Biolayer interferometry (BLI) experiments were performed on an OctetRed 384 system (Fortebio). Amine reactive biosensors (Fortebio) were used to immobilize each of linear, K63-linked and K48-linked Ub_4 at 0.25 μM and various concentrations (from 10 nM to 10 μM) of GST-Rabex-5_{9-73} and its mutants were tested for their binding to the immobilized Ub_4. Sensors were pre-wetted in MES pH 5.5, followed by activation of the pre-wetted sensors with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide. Each of 0.25 μM linear, K63-linked and K48-linked Ub_4 was then coupled to the activated sensors, followed by quenching with 1 M ethanolamine pH 8.5. The immobilized sensors were equilibrated with buffer C (50 mM Tris–HCl pH 7.5, 300 mM NaCl, and 0.05% (v/v) Tween-20), reacted with various concentrations (from 10 μM to 10 nM) of GST-Rabex-5_{9-73} and its mutants, and moved to the buffer C. 10 μM GST was used for the reference sensor. Steady state analysis in Data Analysis version 7.0 (Fortebio) was used to calculate the K_d.

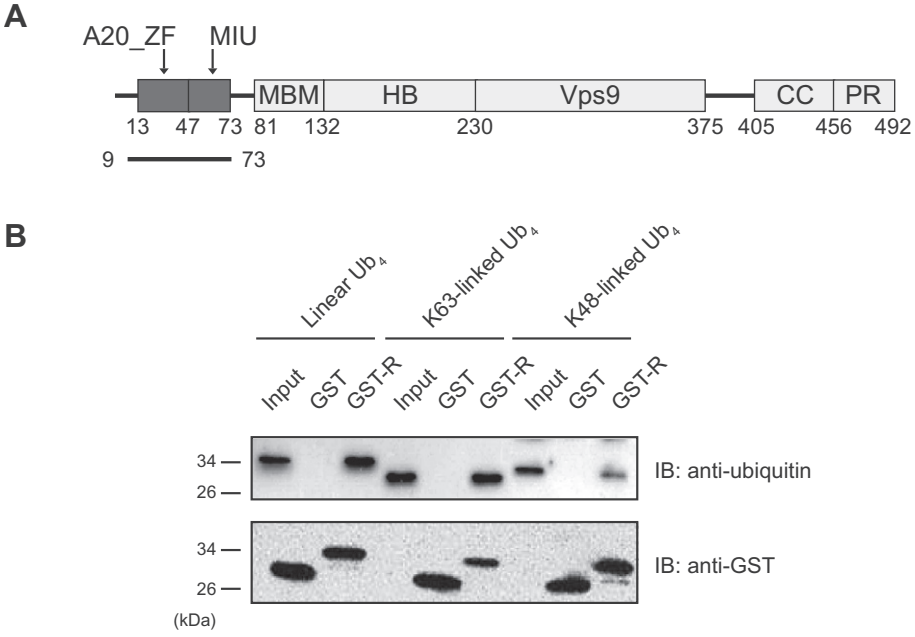

Fig. 1. (A) Domain architecture of Rabex-5. A20_ZF, A20-type zinc finger; MIU, motif-interacting with ubiquitin; MBM, membrane binding motif; HB, helical bundle; Vps9, vacuolar protein sorting 9; CC, coiled coil; PR, proline-rich region. Residue numbers for the boundaries of the domains are shown. The construct which was used for this study is shown as black line at the bottom with starting and ending residue numbers. (B) GST pull-down assay to probe linkage-specific polyubiquitin recognition by Rabex-5_{9-73}. Denaturing gel electrophoresis was performed followed by immunoblotting with antibodies specified.

3. Results

3.1. Recognition of linkage-specific polyubiquitin chains by Rabex-5_{9-73}

To explore systematically how the tandem UBDs of bovine Rabex-5 recognize linkage-specific polyubiquitin chains, we used the N-terminal fragment containing A20_ZF and MIU, termed Rabex-5_{9-73} (Fig. 1A). We performed GST pull-down assay probed by immunoblotting with GST-Rabex-5_{9-73} as the bait and three linkage-specific Ub_4 chains (linear, K63-linked and K48-linked ones) as the preys (Fig. 1B). Rabex-5_{9-73} binds all three linkage-specific Ub_4 tested. It seems that Rabex-5_{9-73} slightly prefers linear and K63-linked Ub_4 over K48-linked one. It was previously demonstrated that A20_ZF-MIU of human Rabex-5 binds K48-linked Ub_{2-7} [18]. Our results indicate that A20_ZF-MIU of Rabex-5 can recognize not only previously known K48-linked polyubiquitin chains but also at least two other linkage-specific ones – linear and K63-linked – in vitro. Together, we establish that the tandem UBDs of Rabex-5 binds both mono- and polyubiquitin.

3.2. Contribution of each UBD of Rabex-5_{9-73} in linkage-specific poyubiquitin recognition

To further investigate the contribution of each UBD of Rabex-5_{9-73} in binding three kinds of linkage-specific Ub_4 chains, we used wild-type (WT), A58D mutant abrogating ubiquitin binding capability of MIU, Y25A/Y26A double mutant that of A20_ZF and Y25A/Y26A/A58D triple mutant that of both UBDs. Sequence alignment of A20_ZF with ZnF4 reveals marginal differences (Fig. 2A). Chemical properties of some residues in weak ubiquitin sites of ZnF4 are reversed in A20_ZF: positively charged Lys-606 of ZnF4 is replaced by hydrophobic Leu-18 in A20_ZF and hydrophobic Leu-626 and Ile-629 by positively charged Lys-37 and Arg-40. To assess the effects of the fore-mentioned residues on polyubiquitin binding, we also prepared a sextuple mutant L18K/K37L/R40I/Y25A/Y26A/A58D based on the sequence alignment between ZnF4 of A20 and A20_ZF of Rabex-5. ZnF4 of A20 was shown to possess one relatively strong, canonical ubiquitin binding site along with two novel and weaker ones [20]. The sextuple mutant was designed to assess whether the weaker ubiquitin binding sties found in ZnF4 of A20 can compensate linkage-specific polyubiquitin binding by Rabex-5_{9-73} in the background of Y25A/Y26A/A58D, the triple mutant. We then performed GST pull-down assays using the three linkage-specific Ub_4 (Fig. 2B). For all three kinds of linkage-specific Ub_4, the A58D mutant completely abrogated polyubiquitin binding, implicating that the MIU domain is critical in linkage-specific polyubiquitin recognition. In contrast, the Y25A/Y26A mutant retained polyubiquitin binding capability albeit the decreased apparent affinity. Such results suggest that the A20_ZF may be a minor contributor in binding linkage-specific polyubiquitin chains and reinforce the finding that the MIU is critical in polyubiquitin binding. Unlike ZnF4 of A20, A20_ZF of Rabex-5 clearly

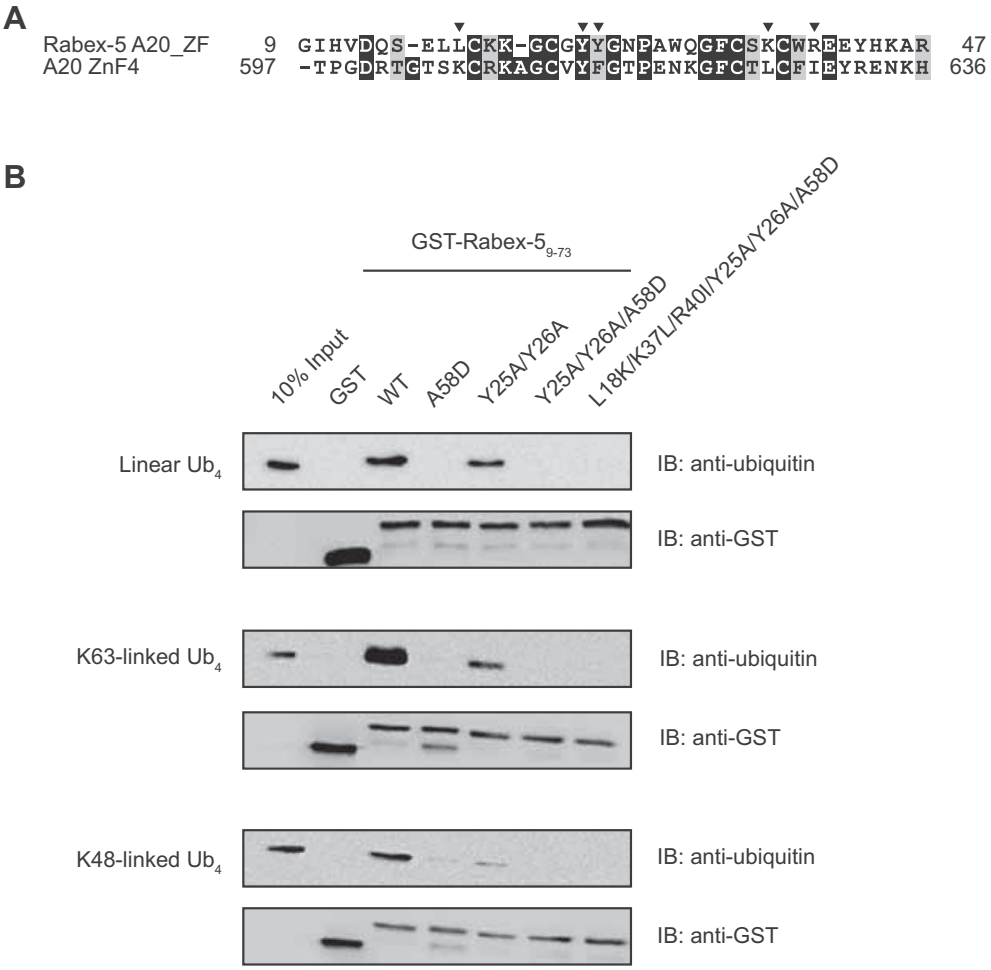

Fig. 2. (A) Sequence alignment of A20_ZF in Rabex-5 with ZnF4 in A20. Alignment was performed using ClustalW [27]. Mutations used for this study are indicated by inverse triangles. (B) Mutational analysis of linkage-specific polyubiquitin recognition by GST-Rabex-5_{9-73}. Each mutant of GST-Rabex-5_{9-73} was immobilized on glutathione-Sepharose beads (GE HealthCare) and linkage-specific Ub_4 chains were added as indicated, washed and analyzed by immunoblotting with specified antibodies.

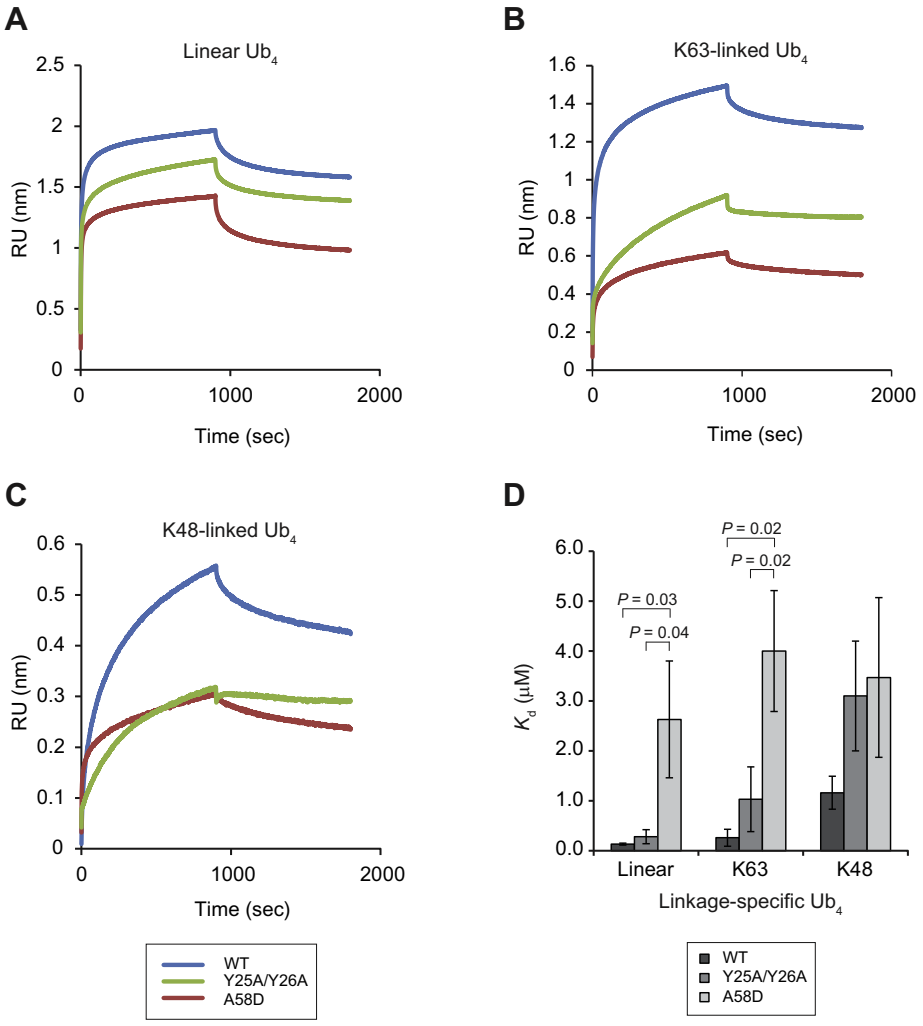

Fig. 3. Quantitative analysis of linkage-specific polyubiquitin recognition by GST-Rabex-5_{9-73} using BLI. Linear (A), K63-linked (B) and K48-linked (C) Ub_4 chains were immobilized on a sensor chip by amine coupling, followed by addition of each mutant of GST-Rabex-5_{9-73} as indicated. (D) Comparison of K_d values derived from BLI analysis. *P* values were calculated by Student's *t*-test.

binds linear and K48-linked Ub_4 as well as K63-linked one. The triple mutant (Y25A/Y26A/A58D) showed no polyubiquitin binding as expected. The sextuple mutant (L18K/K37L/R40I/Y25A/Y26A/A58D) did not compensate the abrogated polyubiquitin binding by the triple mutant, implicating that the weaker ubiquitin binding sites found in ZnF4 of A20 may not contribute to polyubiquitin recognition in Rabex-5. Our data demonstrate that MIU plays a major role in recognizing linkage-specific polyubiquitin chains.

3.3. Quantitative analysis of linkage-specific polyubiquitin recognition by Rabex-5_{9-73}

Next we measured binding affinities of GST-Rabex-5_{9-73} WT, Y25A/Y26A and A58D towards linear, K63- and K48-linked Ub_4 using BLI (Fig. 3A–C and Table 1). A sensor chip with each of linear, K63- and K48-linked Ub_4 immobilized was immersed in solution containing each of GST-Rabex-5_{9-73} mutants (Figs. S1–S3). Rabex-5_{9-73} shows sub μM K_d values towards linear and K63-linked Ub_4 and about 1 μM K_d for K48-linked one (Table 1), indicating that Rabex-5_{9-73} exhibits some selectivity for linear and K63-linked Ub_4 over K48-linked one. MIU has apparent K_d of 0.3–1 μM for linear and K63-linked Ub_4 with a lower K_d value of 3 μM for K48-linked one, inferred by mutational analysis using the Y25A/Y26A double mutant. These results suggest that MIU possesses the same selectivity as Rabex-5_{9-73}. By contrast, A20_ZF exhibits virtually no selectivity for the three linkage-specific Ub_4 chains tested with K_d of 3–4 μM, determined using the A58D mutant (Table 1). Taken together, MIU appears to bind linkage-specific polyubiquitin chains more strongly than A20_ZF with some selectivity. Quantitative BLI data indicate that binding affinities of MIU for linkage-specific Ub_4 chains seem rather high in comparison to a recent report using Ub_2 as a model for polyubiquitin chain [19]. We attribute such discrepancies to different experimental setup between two studies: BLI immobilizes one protein with the other in mobile, solution phase while mass spectrometry deals with ionized, free

Table 1
Binding affinities of Rabex-5_{9-73} to linkage-specific tetraubiquitin chains[a].

Rabex-5_{9-73} mutant	K_d (μM)[b]		
	Linear Ub_4	K63-linked Ub_4	K48-linked Ub_4
WT	0.13 ± 0.02	0.26 ± 0.17	1.2 ± 0.33
Y25A/Y26A	0.28 ± 0.14	1.0 ± 0.65	3.1 ± 1.1
A58D	2.6 ± 1.2	4.0 ± 1.2	3.5 ± 1.6

[a] Each linkage-specific Ub_4 chain was immobilized on a sensor and GST-Rabex-5_{9-73} mutants were added.
[b] K_d values were calculated based on triplicate data and limited to two significant figures.

protein vapors. We cannot rule out the possibility that avidity and/or crowding effect in our experimental set-up may lead to higher-than-expected affinities. However, general correlations for linkage-specific Ub_4 binding of Rabex-5_{9-73} by BLI analysis are reliably reproduced in comparison to those by GST pull-down analysis. Consistent with the GST pull-down data, Rabex-5_{9-73} binds linear and K63-linked Ub_4 more strongly than K48-linked one with statistical significance ($P < 0.05$) (Fig. 3D). A20_ZF appears to bind all three linkage-specific Ub_4 chains with similar but reduced affinities. By contrast, MIU apparently favors linear and K63-linked Ub_4 over K48-linked one. Our data suggest that MIU may be primary site for linkage-specific polyubiquitin chains with A20_ZF being a secondary one.

4. Discussion

The finding that A20_ZF of Rabex-5 recognizes not only K63-linked polyubiquitin but also linear and K48-linked one is in clear contrast to orthologous ZnF4 of A20. ZnF4 binds preferentially K63-linked Ub_3 with much less affinities for linear, K48- and K11-linked Ub_3 [20]. The sextuple mutant contains residues from the weaker binding site of ZnF4 (L18K/K37L/R40I) while the conserved residues in the strong binding site are knocked down (Y25A/Y26A/A58D). We observed no increase in polyubiquitin binding by the sextuple mutant in comparison to that by the triple mutant (Fig. 2B), which implicates that weaker ubiquitin binding sites identified in ZnF4 are unlikely to contribute to polyubiquitin recognition by Rabex-5. For comparison, A20_ZF of a plant protein, stress associated protein 5 from *Arabidopsis thaliana*, exhibits similar linkage-specific polyubiquitin selectivity to that of Rabex-5 [21] . It is plausible that differences in linkage-specific polyubiquitin selectivity between ZnF4 of A20 and A20_ZF of Rabex-5 may lie in the residues involved in the weak ubiquitin binding sites in ZnF4.

The present study suggests that MIU may be more important in recognizing linkage-specific polyubiquitin chains than A20_ZF. It is tempting that Rabex-5 may use MIU for binding polyubiquitin moiety of a target protein and A20_ZF for functions other than polyubiquitin recognition. It was previously noted that Rabex-5 is recruited to endosomes dependent of ubiquitin binding by the two UBDs [22]. Interestingly, the Y25A/Y26A mutant, although it is predominantly localized in cytosol, can associate with endosomes [22], supporting the idea that MIU is the main UBD for polyubiquitin recognition in Rabex-5. In parallel with that A20_ZF may be utilized for functions other than polyubiquitin binding in vivo, we observed that monoubiquitinated L1 neural adhesion molecule is endocytosed in a strictly MIU-dependent manner in cultured cells (unpublished data). Considering that A20_ZF of Rabex-5 possesses an E3 ubiquitin ligase activity [14,15,18] with its substrate specificity toward Ras proteins [16,17], the ubiquitin binding capability of A20_ZF may be for functions other than simple ubiquitin recognition *per se*.

A few cases have been reported for biochemical characterization of tandem ubiquitin binding domains in polyubiquitin recognition. RIG-I recognizes K63-linked polyubquitin chains by its tandem CARD domains spanning about 200 residues at the N-terminus [23]. RAP80 binds selectively linear and K63-linked polyubiquitin and such recognition is mediated by tandem UIM domains [8,9,24–26]. In Rabex-5, two UBDs are fused to constitute one continuous structural entity whereas RIG-I and Rap80 have connecting linker between two identical UBDs. Our study provides an instance of polyubiquitin recognition by heterologous tandem ubiquitin binding domains.

Here we systematically investigated linkage-specific polyubiquitin recognition by the tandem UBDs of Rabex-5, A20_ZF and MIU, by qualitative and quantitative techniques in vitro. A20_ZF-MIU (Rabex-5_{9-73}) recognizes linear, K63- and K48-linked Ub_4 with slight preferences to linear and K63-linked ones. We also demonstrated that Rabex-5_{9-73} shows K_d of 0.1–1 μM towards linear and K63-linked Ub_4. Mutational analysis reveals that MIU appears to be more important than A20_ZF when Rabex-5_{9-73} binds polyubiquitin chains, which is supported by both qualitative and quantitative data. Our study establish that the tandem UBDs in Rabex-5 bind both mono- and polyubiquitin and that Rabex-5 exhibits moderate selectivity for different linkage-specific polyubiquitin chains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.032.

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