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Cooperative and selective roles of the WW domains of the yeast Nedd4-like ubiquitin ligase Rsp5 in the recognition of the arrestin-like adaptors Bul1 and Bul2

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ABSTRACT

The ubiquitin ligase Rsp5, which is the only yeast *Saccharomyces cerevisiae* member of the Nedd4-family, recognizes and ubiquitinates various substrate proteins through the functions of three conserved WW domains. To elucidate the role of each WW domain in endocytosis of the general amino acid permease Gap1 via interaction with the arrestin-like adaptor proteins Bul1 and Bul2 (Bul1/2), we investigated the effects of the double mutations that abrogate the recognition of PY motifs on target proteins (*rsp5*$^{W257F/P260A}$, *rsp5*$^{W359F/P362A}$, and *rsp5*$^{W415F/P418A}$) and the alanine substitutions of the conserved threonine residues that are regarded as putative phosphorylation sites (*rsp5*^{T255A}, *RSP5*^{T357A}, and *rsp5*^{T413A}), both of which are located within each WW domain. The *rsp5*$^{W257F/P260A}$, *rsp5*$^{W359F/P362A}$, and *rsp5*$^{W415F/P418A}$ mutations increased sensitivity to the proline analog azetidine-2-carboxylate (AZC), defective endocytosis of Gap1, and impaired interactions with Bul1. These results demonstrate that molecular recognition by each WW domain is responsible for the cooperative interaction with Bul1. Intriguingly, the *RSP5*^{T357A} mutation enhanced AZC tolerance and endocytosis of Gap1, although *rsp5*^{T255A} and *rsp5*^{T413A} decreased both of them. While *rsp5*^{T255A}, *RSP5*^{T357A}, and *rsp5*^{T413A} impaired the interaction of Rsp5 with Bul1, the *RSP5*^{T357A} mutation specifically augmented the interaction with Bul2. The AZC tolerance enhanced by *RSP5*^{T357A} was fully abolished by combining with each of the *rsp5*$^{W257F/P260A}$, *rsp5*$^{W359F/P362A}$, or *rsp5*$^{W415F/P418A}$ mutations. It was thus suggested that Thr357 in the WW2 domain has a unique role in preventing from the constitutive activation of Bul1/2-mediated endocytosis of Gap1. Taken together, our results highlight the cooperative and specific roles of WW domains in the regulation of Bul1/2-mediated cellular events.

1. Introduction

The yeast *Saccharomyces cerevisiae* ubiquitin ligase Rsp5 and its mammalian homologue Nedd4 play essential roles in the physiological control of diverse processes, such as intracellular trafficking, transcriptional regulation, cell signaling, and quality control of cytosolic and plasma membrane proteins [1–4], through direct interaction with a wide variety of substrate proteins. The Nedd4-family ubiquitin ligases commonly contain an amino-terminal C2 domain involved in membrane phospholipid binding, two to four WW domains (referring to a pair of conserved tryptophan (W) residues) that bind to proline-rich PY motifs in substrate proteins, and a carboxyl-terminal homologous to the E6-AP carboxyl terminus (HECT) catalytic domain [1,2]. Among them, the protein–-protein interacting WW domains are believed to determine the substrate specificity of Rsp5 and Nedd4.

Several amino acid residues in WW domains are responsible for the interaction with the ligand proteins. The second tryptophan and the nearby proline residues (WXXP sequence) highly conserved in each WW domain (Fig. 1) are important for *in vitro* binding with a proline-rich ligand [5,6], and thus conversion of WXXP into AXXP, AXXA, or FXXA is used to disrupt its binding activity. In the *S. cerevisiae* Rsp5, such mutation in the WW1 or 3 domain severely impairs fluid-phase endocytosis [7,8], while the

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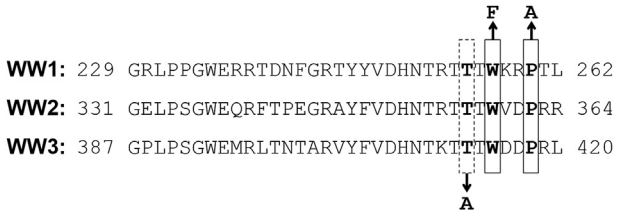

Fig. 1. Amino acid sequence alignment of the Rsp5 WW domains. The conserved tryptophan and proline residues in the WXXP sequence and the putative threonine phosphorylation sites in Rsp5 are shown in solid boxes and a dashed box, respectively. The arrows show the amino acid substitutions performed in this study.

WW2 and 3 domains are important for sorting the cargoes into multivesicular bodies (MVBs) [9,10] and activating homologous transcription factors Spt23 and Mga2 [11]. The WW2 domain also presents a predominant function in interaction with the large subunit of RNA polymerase II [12]. However, the redundant and specific roles of Rsp5 WW domains are not fully understood.

Our previous random mutagenesis of Rsp5 revealed that the conserved threonine residue in the WW2 domain (Thr357) has a negative role in endocytosis of the general amino acid permease Gap1 [13,14]. The alanine substitution of Thr357 (*RSP5*^{T357A}) leads to constitutive internalization of Gap1 into the vacuole, and thus enables cells to grow in the presence of a toxic proline analog, azetidine-2-carboxylate (AZC) [14]. Phosphorylation of the corresponding threonine residue (Thr30) in mouse Itch, which belongs to the Nedd4-family, inhibits *in vitro* binding to the PY motif-containing ligands [15]. Consistently, analyses of the *RSP5* mutants using an anti-phosphorylated WW domain antibody supported the idea that Thr255, Thr357, and Thr413 in the WW1, 2, and 3 domains, respectively, are *in vivo* phosphorylation sites [14]. Although *RSP5*^{T357A} confers high tolerance to AZC, the *rsp5*^{T255A} and *rsp5*^{T413A} substitutions inhibit growth in the presence of AZC [14], suggesting that the phosphorylation of individual WW domains has distinct functions in the endocytosis of Gap1. Given that the PY motif-containing arrestin-like adaptor proteins Bul1 and Bul2 (Bul1/2) [16,17] mediate ubiquitination and downregulation of Gap1 by Rsp5 [18,19], the threonine residue of each WW domain may play different roles in the interaction with Bul1/2. More recently, we also identified the *RSP5*^{P343S} mutation in the WW2 domain, which specifically increases the affinity for ectopically expressed human α-synuclein in yeast cells [20].

In this study, we investigated the effects of amino acid substitutions in individual WW domains on endocytosis of Gap1 via interaction with Bul1/2. This study will help us to understand how each WW domain specifically contributes to determining the substrate specificity of Rsp5 and related ubiquitin ligases conserved among higher eukaryotes.

2. Materials and methods

2.1. Strains, plasmids, and culture media

All yeast strains used in this study were the *S. cerevisiae* strains with a BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) background (provided by EUROSCARF). Strains Δ*rsp5* [pAD4-OLE1], Δ*bul1*, Δ*bul2*, *rsp5*^{T255A}, *RSP5*^{T357A}, *RSP5*^{T357A} Δ*bul1*, *RSP5*^{T357A} Δ*bul2*, and *rsp5*^{T413A} were previously constructed [14]. Plasmid pAD4-OLE1 was used to overexpress the *OLE1* gene and suppress the lethality of *RSP5* deletion [21]. When appropriate, required empty vectors harboring the *HIS3*, *LEU2*, *MET15*, or *URA3* gene were introduced to complement the auxotrophy. *Escherichia coli* strain DH5α and DB3.1 (Invitrogen) were used to construct vectors or subclone the yeast genes.

The oligonucleotide primers are listed in Table S1 of Supplementary Material. The *rsp5*$^{W257F/P260A}$, *rsp5*$^{W359F/P362A}$, or *rsp5*$^{W415F/P418A}$ mutation was introduced into plasmids pRS416-RSP5 and pRS416-RSP5^{T357A} [14] using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) and oligonucleotide primer pairs RSP5-WW1-FA-Fw and RSP5-WW1-FA-Rv, RSP5-WW2-FA-Fw and RSP5-WW2-FA-Rv, and RSP5-WW3-FA-Fw and RSP5-WW3-FA-Rv, respectively. To introduce the *rsp5*$^{W359F/P362A}$ mutation into plasmid pRS416-RSP5^{T357A}, primers RSP5-WW2-T357A-FA-Fw and RSP5-WW2-T357A-FA-Rv were used instead. To delete the C2 domain of pRS416-RSP5, a PCR product using pRS416-RSP5 as a template and primers RSP5-C2-truncate-Fw and RSP5-C2-truncate-Rv was 5′-phosphorylated by T4 polynucleotide kinase and self-ligated to generate pRS416-RSP5ΔC2. The *RSP5*^{T357A} mutation was introduced into this plasmid as previously described [14] to construct pRS416-RSP5^{T357A}ΔC2. Plasmid pAG413-P_{GAL1}-GAP1-yEGFP-T_{CYC1} was produced via LR reactions between pDONR221-GAP1 [14] and pAG413-P_{GAL1}-ccdB-yEGFP-T_{CYC1} (Addgene). Plasmids pAG423-P_{GPD}-yEGFP-BUL1-T_{CYC1} and pAG423-P_{GPD}-yEGFP-BUL2-T_{CYC1} were produced via LR reactions between pDONR221-BUL1 or pDONR221-BUL2 [14] and pAG423-P_{GPD}-yEGFP-ccdB-T_{CYC1} (Addgene), respectively. Plasmids pAG416-P_{GPD}-yEGFP-BUL1-T_{CYC1} and pAG416-P_{GPD}-yEGFP-BUL2-T_{CYC1} were previously constructed [14].

Disruption of the *BUL1* or *BUL2* gene in the *rsp5*^{T255A} and *rsp5*^{T413A} strains was conducted as previously described [14]. To construct Δ*rsp5* strains with pRS416-RSP5, pRS416-rsp5ΔC2, pRS416-RSP5^{T357A}, or pRS416-RSP5^{T357A}ΔC2, each plasmid was introduced into BY4741, and then, the original *RSP5* gene on the chromosome was disrupted by a PCR-based gene disruption method. A disruption cassette amplified by PCR with pFA6-hphNT1 [22] using oligonucleotide primers RSP5-S1 and RSP5-S2 was integrated into the *RSP5* locus. Gene disruptants were selected on YPD medium containing hygromycin B. Correct disruption was confirmed by genomic PCR using primers RSP5-up and RSP5-down.

The media used for growth of *S. cerevisiae* were a nutrient medium YPD (1% yeast extract, 2% peptone, and 2% glucose) and a synthetic minimal medium SD (0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose) containing 0.5% ammonium sulfate or 0.1% allantoin as the sole nitrogen source. For galactose induction, SG (0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% galactose) was used. Yeast strains were also cultured on SD agar plates containing indicated concentrations of AZC. *E. coli* cells were grown in Luria–Bertani (LB) medium containing 100 μg/mL ampicillin or 50 μg/mL kanamycin.

2.2. Fluorescent microscopy

Yeast cells carrying pAG413-P_{GAL1}-GAP1-yEGFP-T_{CYC1} were grown to an OD_{600} of 1 in SG − N + allantoin liquid medium to induce the expression of Gap1-GFP, and the cells were washed with distilled water and incubated in SD − N + allantoin liquid medium for 3 h to shut off the induction. Distilled water or 50 mM ammonium sulfate was added to each culture, and the cells with Gap1-GFP signal were observed 1 h later under a fluorescence microscope Axiovert 200M (Carl Zeiss). Images were captured with a HBO 100 Microscope Illuminating System (Carl Zeiss) digital camera.

2.3. Co-immunoprecipitation and Western blot

Yeast cells carrying pAG423-P_{GDP}-yEGFP-BUL1-T_{CYC1} or pAG423-P_{GDP}-yEGFP-BUL2-T_{CYC1} (for Δ*rsp5* [pAD4-OLE1] strains with pRS416-RSP5, pRS416-rsp5$^{W257F/P260A}$, pRS416-rsp5$^{W359F/P362A}$, or pRS416-rsp5$^{W415F/P418A}$) or pAG416-P_{GDP}-yEGFP-BUL1-T_{CYC1} or pAG416-P_{GDP}-yEGFP-BUL2-T_{CYC1} (for BY4741 wild type, *rsp5*^{T255A}, *RSP5*^{T357A}, and *rsp5*^{T413A}) were grown in SD − N + allantoin liquid

medium to an OD_{600} of 1. One hour after 50 mM ammonium sulfate was added to each culture, cells were harvested, suspended in iced lysis buffer [20 mM Tris–HCl (pH8.0), 150 mM sodium chloride, 10% glycerol, 0.5% NP-40, and protease inhibitors], and lysed with glass beads. After centrifugation (10 min at 3000 rpm), supernatants were incubated with 10 μL of equilibrated anti-GFP magnetic beads (Medical & Biological Laboratories) for 1 h at 4 °C. The beads were washed three times with wash buffer [50 mM Tris–HCl (pH8.0), 150 mM sodium chloride, 10% glycerol, and 0.1% NP-40]. Immunoprecipitates were solubilized by incubation in fivefold concentration of sample buffer [250 mM Tris–HCl (pH6.8), 10% sodium dodecyl sulfate (SDS), 11.25% glycerol, 25% 2-mercaptoethanol, and 0.0625% bromophenol blue] for 5 min at 95 °C. Each sample was loaded on an 8% SDS-polyacrylamide gel. The Rsp5, GFP-Bul1/2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins were detected by an anti-Rsp5 mouse antibody (provided by Jon Huibregste) at 1:10,000 dilution, an anti-GFP mouse antibody (Roche) at 1:2000 dilution, and an anti-GAPDH rabbit antibody (Nordic Immunological Laboratories) at 1:10,000 dilution, respectively.

3. Results

3.1. Roles of conserved WXXP sequences in endocytosis of Gap1 via interaction with Bul1/2

To identify which WW domains are specifically involved in endocytosis of Gap1, we first examined the effects of mutation in the WXXP sequence that is highly conserved among WW domains (Fig. 1). Growth in the presence of the toxic proline analog AZC under rich nitrogen sources (e.g., ammonium) is highly correlated to downregulation of cell-surface amino acid permeases, such as Gap1. We previously revealed that in the *rsp5*^{A401E} strain having a loss-of-function mutation in the *RSP5* gene, Gap1 remains stable and active on the plasma membrane, without ubiquitination, leading to hypersensitivity to AZC [23]. As clearly shown in Fig. 2A, the conversion of WXXP into FXXA in each WW domain led to decreased tolerance even to a low concentration (10 μM) of AZC. Among the tested double mutations, *rsp5*$^{W359F/P362A}$ exhibited the weakest defect in the growth on the AZC-containing medium. Consistently, all FXXA mutants showed severe defects in endocytic sorting of Gap1-GFP to the vacuole (Fig. 2B). Although the percentage of cells with vacuolar-localized Gap1-GFP was greatly elevated in the wild-type strain (BY4741) after addition of ammonium sulfate to the medium, there was no significant increase in the *rsp5*$^{W257F/P260A}$ or *rsp5*$^{W415F/P418A}$ cells with vacuolar Gap1-GFP. Only a portion of the *rsp5*$^{W359F/P362A}$ cells exhibited intense vacuolar localization of Gap1-GFP upon the switching of nitrogen sources. These results suggest that the recognition of PY motifs by individual WW domains is essential, although the importance of the WW2 domain is relatively low, for endocytosis of Gap1.

Since Gap1 does not contain typical PY motifs, the PY motif-containing adaptor proteins Bul1/2 are required for ubiquitination and endocytosis of Gap1 [18,19]. To examine whether the defective endocytosis of Gap1 observed in the FXXA mutants is attributable

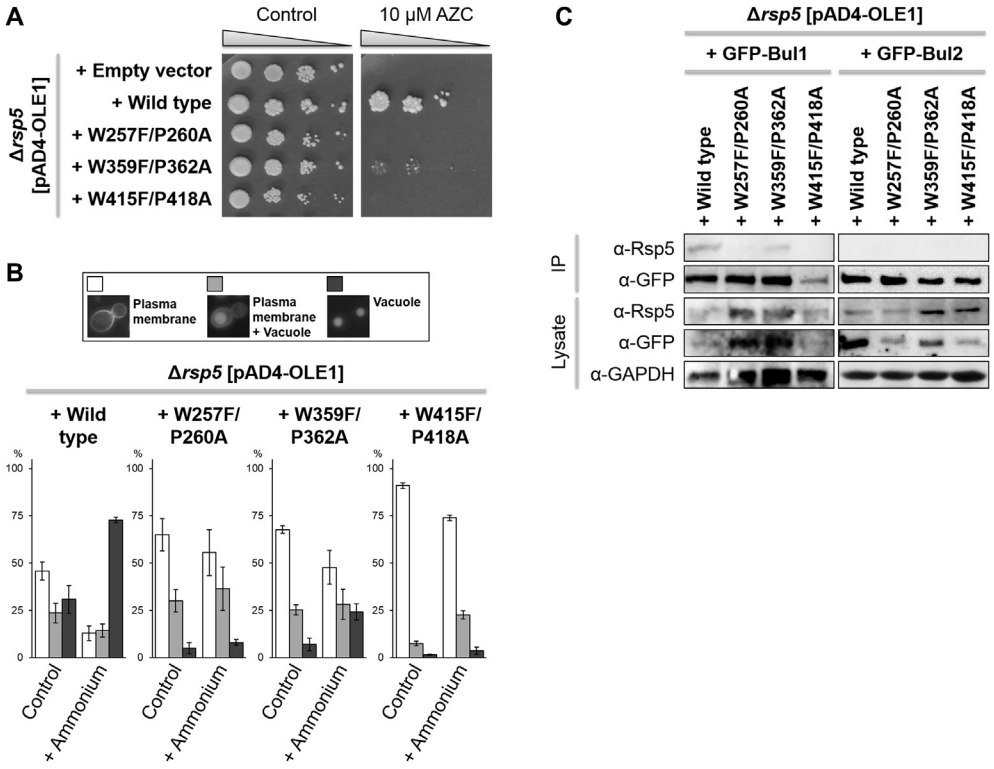

Fig. 2. Effects of the binding activity of the WW domains toward PY motifs. (A) AZC tolerance test. Each strain was grown to the logarithmic growth phase in SD − N + allantoin liquid medium. Approximately 10^7 cells (OD_{600} of 1) of each strain and serial dilutions of 10^{-1}–10^{-3} (from left to right) were spotted onto SD agar medium in the absence (left panel) and presence of AZC (right panel). The plates were incubated at 25 °C for 3 d. (B) Microscopic observation of Gap1-GFP fusion protein. Each strain was grown to an OD_{600} of 1 in SG − N + allantoin liquid medium to induce the expression of Gap1-GFP, and the cells were washed with distilled water and incubated in SD − N + allantoin liquid medium for 3 h to shut off the induction. Distilled water (control) or 50 mM ammonium sulfate (+ammonium) was added to each culture, and the cells with Gap1-GFP signal in the plasma membrane (white bars), in both the plasma membrane and the vacuole (light gray bars), and in the vacuole (dark gray bars) were counted 1 h later. The values are the means and standard deviations of three independent experiments with more than 100 cells counted. (C) Interaction between Rsp5 and Bul1/2. The cells expressing GFP-Bul1 (left panels) or GFP-Bul2 (right panels) were grown in SD − N + allantoin liquid medium to an OD_{600} of 1. Whole cell lysates were prepared at 1 h after addition of 50 mM ammonium sulfate to each culture and subjected to co-immunoprecipitation assay using an anti-GFP antibody (top two panels), followed by Western blot analysis using anti-Rsp5 (α-Rsp5), anti-GFP (α-GFP), and anti-GAPDH (α-GAPDH) antibodies.

to the impaired interaction between Rsp5 and Bul1/2, we immunoprecipitated overexpressed GFP-Bul1 or GFP-Bul2 from the cells after addition of ammonium sulfate, and analyzed co-immunoprecipitated Rsp5 (Fig. 2C). As a result, we detected only wild-type Rsp5 and its Rsp5$^{W359F/P362A}$ variant interacting with GFP-Bul1, with a weaker signal of the Rsp5$^{W359F/P362A}$ variant. Therefore, this result supports the model in which each WW domain has a crucial role in binding to Bul1 for ubiquitination and endocytosis of Gap1. No significant interaction was detected between Rsp5 and GFP-Bul2, suggesting that Rsp5 has higher affinity for the Bul1 molecule than the Bul2 molecule *in vivo*, although both Bul1/2 have redundant functions toward endocytosis of Gap1 [14,18,19].

3.2. Roles of conserved threonine residues in endocytosis of Gap1 via interaction with Bul1/2

Next, we focused on the functions of conserved threonine corresponding to the second residue amino-terminal to the second conserved tryptophan residue in the WW domain, which is assumed to be a potential phosphorylation site (Fig. 1) [14]. It is intriguing that the *rsp5*^{T255A} and *rsp5*^{T413A} cells were hypersensitive to AZC, while the *RSP5*^{T357A} mutation conferred strong tolerance toward a high concentration of AZC (1 mM) (Fig. 3A), as previously shown [14], suggesting that these threonine residues have distinctive roles in the endocytosis of Gap1. In agreement with this idea, endocytosis of Gap1-GFP after shifting to a rich nitrogen source was severely inhibited in the *rsp5*^{T255A} and *rsp5*^{T413A} mutants, although it was constitutively enhanced in the *RSP5*^{T357A} mutant (Fig. 3B). Therefore, these results demonstrate that Thr255 and Thr413 are necessary for endocytosis of Gap1, and Thr357 has a specific negative role in endocytosis of Gap1.

Co-immunoprecipitation analysis of GFP-Bul1 revealed that the alanine substitutions commonly decreased the interaction with Bul1 (Fig. 3C, left panels). An amino acid change in the WW1 and WW3 domains severely affected the interaction, while Thr357Ala in the WW2 domain only slightly impaired, as observed in the FXXA mutants (Fig. 2C). Although the GFP-Bul2-interacting Rsp5 was hardly detected in the wild-type and the *rsp5*^{T255A} and *rsp5*^{T413A} mutants, the *RSP5*^{T357A} mutation remarkably enhanced the interaction with Bul2 (Fig. 3C, right panels). Based on these data, it is likely that the conserved threonine residues are commonly required for the interaction with Bul1, and only Thr357 has a unique negative role in the interaction with Bul2.

In addition, a single FXXA mutation in each WW domain fully canceled the high AZC tolerance of the *RSP5*^{T357A} mutant (Fig. 4A). In contrast, deletion of the amino-terminal C2 domain, which is involved in the internalization of Gap1 in an ubiquitination-independent manner [24], did not affect the AZC tolerance (Fig. 4B). This result also supports the idea that the direct interaction between Rsp5 and Bul1/2 and concomitant ubiquitination of Gap1 are responsible for the constitutively activated endocytosis of Gap1 in the *RSP5*^{T357A} mutant.

4. Discussion

The Nedd4-family ubiquitin ligases often contain more than one WW domain. To elucidate the redundancy and the specificity of the three WW domains of the *S. cerevisiae* Rsp5 in the endocytic control of the amino acid permease Gap1, we analyzed the effects of amino acid changes (conversion of the conserved WXXP sequences into FXXA) in individual WW domains. The results showed that all WW domains must be functional to stably interact with Bul1 and to cause endocytosis of Gap1 in response to the switching of nitrogen sources (Fig. 2). Among the three WW domains, the WW2 domain seemed less essential. Despite the unsuccessful detection of Rsp5 co-precipitating with GFP-Bul2, it has been genetically shown that Bul2 has a redundant function with Bul1 in the internalization of Gap1 and subsequent AZC tolerance [14,18,19]. Thus, weak or transient interactions between Rsp5 and Bul2 might be similarly impaired by the FXXA mutations. In sum, we here established a cooperative role of three WW domains in the endocytosis of Gap1 via interaction with Bul1/2.

Although a single WW domain is sufficient to directly interact with a PY motif *in vitro* [5,6], Rsp5 and its mammalian homologue Nedd4 require plural WW domains to exert their full functions

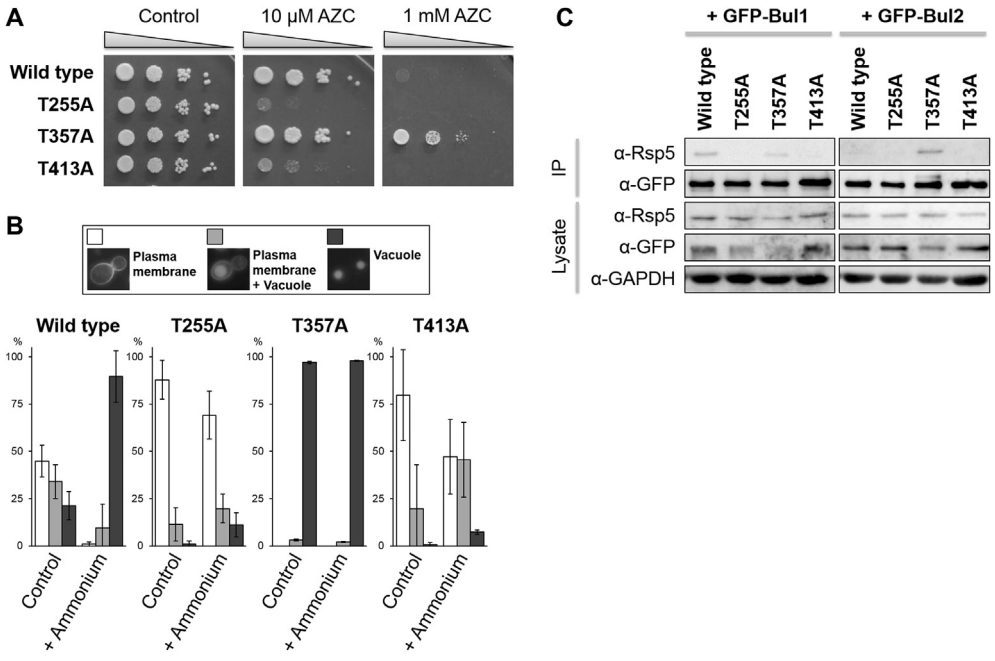

Fig. 3. Effects of the alanine substitutions of conserved threonine residues in the WW domains. (A) AZC tolerance test. (B) Microscopic observation of Gap1-GFP fusion protein. (C) Interaction between Rsp5 and Bul1/2. For the other conditions and information, please refer to Fig. 1.

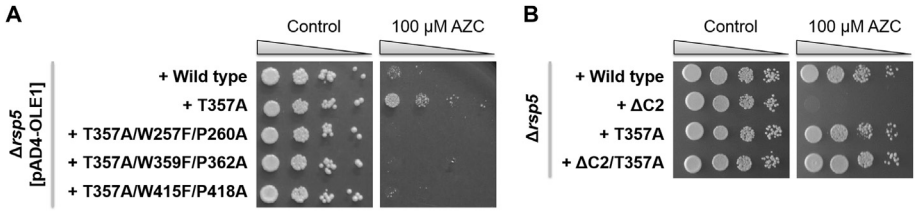

Fig. 4. Interaction between the *RSP5*^{T357A} mutation and the loss-of-function mutations in the WW or C2 domain. (A) Genetic interaction between the *RSP5*^{T357A} mutation and the loss-of-function mutations in the WW domains. (B) Genetic interaction between the *RSP5*^{T357A} mutation and the deletion mutation of the C2 domain.

toward most substrates. For example, the endocytosis of other yeast plasma membrane permeases, such as Fur4 and Can1, is abrogated by disruption of the WW2 or WW3 domain [8,25]. Ubiquitination-dependent internalization of the yeast α-factor receptor Ste2 is impaired by a loss-of-function mutation in each of the three WW domains [7]. The two WW domains of mammalian Nedd4 or Nedd4-2 cooperatively recognize and downregulate epithelial sodium channels [26,27]. To understand this combinational action of WW domains in substrate recognition, their possible interaction with substrate amino acid sequences other than the PY motifs might be considered. Given that WW domains can also bind to phosphoserine and phosphothreonine residues [28], more than one WW domain might form a stable complex with a single substrate molecule through interactions with the PY motifs and other unidentified residues on Bul1/2. Alternatively, Rsp5 and Bul1/2 molecules might form a stable multimeric complex *in vivo* because Rsp5 has been reported to interact with itself [7]. To understand the cooperative behaviors of the WW domains, the native Rsp5-Bul1/2 complex and its dynamics should be analyzed.

In this study, we also focused on the roles of the conserved threonine residues (Thr255, Thr357, and Thr413) that are hypothesized to be phosphorylation sites in the individual WW domains of Rsp5 [14]. Since *in vitro* phosphorylation of a corresponding threonine residue in the WW3 domain of mammalian Itch inhibits the interaction with the PY motif-containing ligands [15], the alanine substitution of these threonine residues might allow the constitutive binding of substrate proteins. The *RSP5*^{T357A} mutation in the WW2 domain causes constitutive vacuolar sorting of Gap1, leading to high AZC tolerance [14]. In contrast, the *rsp5*^{T255A} and *rsp5*^{T413A} mutations in the WW1 and WW3 domains, respectively, decreased the functions of Rsp5 toward Gap1 via Bul1/2 (Fig. 3). Thus, we revealed specific novel roles of these threonine residues in the endocytic control of Gap1. We recently re-identified the *rsp5*^{T255A} mutation, which alleviates the toxicity of overexpressed human α-synuclein or a high concentration of acetate [20], while the *RSP5*^{T357A} and *rsp5*^{T413A} mutants do not exhibit such phenotypes (D. Watanabe et al., unpublished result). These results also support the notion that alteration of a threonine residue at position 255, 357, or 413 affects the specific ability in substrate recognition.

Intriguingly, all the tested alanine substitutions decreased the interaction with Bul1, as the FXXA mutations did, although the *RSP5*^{T357A} mutation specifically increased the interaction with Bul2 (Fig. 3C). This is the first observation suggesting that there are distinct mechanisms for the recognition of redundant Bul1/2 proteins, and might partly account for the specific phenotype of the *RSP5*^{T357A} mutant. It should be noted, however, that disruption of both the *BUL1/2* genes [14] or an FXXA mutation in each of the WW domains (Fig. 4A) is required to abolish the constitutive active phenotype of the *RSP5*^{T357A} mutant [14]. Based on these results, the *RSP5*^{T357A} mutation is suggested to specifically enhance endocytosis of Gap1 via a coordinated action of three WW domains and Bul1/2. To clarify the novel regulatory mechanism of the Rsp5 activity by phosphorylation of Thr357, detection of phosphorylation sites with mass spectrometry and identification of kinases and phosphatases that directly act on Rsp5 should be carried out.

The Rsp5-Bul1/2 complex is involved in the control of various cellular functions, such as intracellular trafficking of permeases including Gap1 [8,18,19,25,29], stress- and/or nutrient-responsive gene expression [17,30,31], and quality control of plasma membrane proteins [3,32]. Cooperative and selective recognition of the Bul1/2 proteins by the WW domains may contribute to fine-tuning of the ubiquitination status of multiple substrate proteins in a coordinated way with extracellular cues. Our findings in this study will help us to understand the molecular mechanisms of substrate recognition by Rsp5 and the other Nedd4-family ubiquitin ligases that are highly conserved among eukaryotes.

Conflict of interest

The authors (Daisuke Watanabe, Hiroki Murai, Ryoya Tanahashi, Keishi Nakamura, Toshiya Sasaki, and Hiroshi Takagi) declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.025.

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