The Journal of Microbiology (2012) Vol. 50, No. 2, pp. 256–262
DOI 10.1007/s12275-012-1326-z

Effects of Mutations in the WD40 Domain of α-COP on Its Interaction with the COPI Coatomer in *Saccharomyces cerevisiae*

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(Received June 30, 2011 / Accepted November 14, 2011)

Replacement of glycine 227 in the fifth WD40 motif of α-COP/Ret1p/Soo1p by charged or aromatic amino acids is responsible for the temperature-dependent osmo-sensitivity of *Saccharomyces cerevisiae*, while truncations of WD40 motifs exerted a reduction in cell growth rate and impairment in assembly of cell-wall associated proteins such as enolase and Gas1p. Yeast two-hybrid analysis revealed that the *ret1-1/soo1-1* mutation of α-COP abolished the interaction with β- and ε-COP, respectively, and that the interaction between α-COP and β-COP relied on the WD40 domain of α-COP. Furthermore, although the WD40 domain is dispensable for interaction of α-COP with ε-COP, structural alterations in the WD40 domain could impair the interaction.

Keywords: α-COP, mutagenesis, *Saccharomyces cerevisiae*, WD40 domain, yeast two-hybrid

Introduction

Intracellular protein transport in eukaryotic cells is mediated by small transport vesicles that are defined by their coat proteins (COPs). COPII-coated vesicles are thought to mediate the bulk flow of proteins from the endoplasmic reticulum (ER) to the early Golgi. COPI vesicles mediate the recycling of proteins from the Golgi to the ER, and clathrin-coated vesicles mediate transport from the *trans*-Golgi network, as well as endocytic transport from the plasma membrane (McMahon and Mills, 2004). In coated vesicle formation, conformational changes of the coat protein complexes activate them for interaction with cargo or machinery components and coat polymerization (Langer *et al.*, 2007).

The COPI coat consists of the small *ras*-like GTPase, GTP-bound ADP ribosylation factor (ARF), and coatomer, which is a stable cytosolic 700–800 kDa complex. Coatomer comprises seven equimolar subunits, α-, β-, β′-, γ-, δ-, ε-, and ζ-COP. Except for ε-COP, which stabilizes α-COP and thus coatomer at elevated temperatures, each of these COP proteins is essential for yeast cell viability. Four interacting pairs of coatomer subunits have been identified by yeast two-hybrid analysis: β/δ-, γ/ζ-, α/ε-, and αβ′-COPs. A stable COPI B-subcomplex composed of α-, β′-, and ε-COP, which interacts with KKXX motifs, is generated by disintegration of coatomer in high salt buffers (Faulstich *et al.*, 1996). Furthermore, Fiedler *et al.* (1996) showed the generation of an α-, β-, γ-, δ-, ζ-COP subcomplex of coatomer, which tends to disassemble further into two stable heterodimers consisting of β/δ- and γ/ζ-COPs (Eugster *et al.*, 2000). Although a tetrameric cargo-binding sub-complex (βδ/γζ-COP) and a trimeric cage-forming sub-complex (αβ′ε-COP) of the coatomer have been identified, far less is known about the architecture of the COPI coat (Lee and Goldberg, 2010).

Across distant species, α- and β′-COP both have a highly conserved N-terminal domain comprising six and five WD40 motifs, respectively (Schröder-Kohne *et al.*, 1998; Chechenova *et al.*, 2004; Eugster *et al.*, 2004). WD40 motifs are conserved sequence motifs predicted to fold into a structure composed of β-strands and turns, the so-called β-propeller structure (Neer and Smith, 2000). It has been suggested that the α-COP WD40 domain may be involved in interaction with COPI vesicle cargo, e.g. KKXX-tagged proteins (Schröder-Kohne *et al.*, 1998; Eugster *et al.*, 2004). Since point mutations in the α-COP WD40 domain cause a broad range of phenotypes, Eugster *et al.* (2000) investigated the role of the N- and C-terminal regions of α-COP *in vivo* using truncation mutants and concluded that the WD40 domain of α-COP is dispensable for yeast cell viability, but is required for KKXX-dependent trafficking and KKXX binding. They also reported that a truncated version of α-COP lacking the entire WD40 domain supports yeast cell viability up to 34°C, but not at 37°C. Interestingly, among the WD40 domain mutations identified to date, mutations in the fifth WD40 motif, such as *ret1-1* (G227D), *ret 1-2* (S226F), and *sec33-1* (P147L, S226F), result in the temperature-sensitive phenotypes. Despite recent progress in the understanding of the molecular mechanisms and function of the COPI system (Beck *et al.*, 2009; Hsia and Hoelz, 2010), its fate and function are still controversial and an understanding of the architecture of the COPI-coated vesicle remains elusive.

Previously, we found that the temperature-sensitive phenotype of the *ret1-1/soo1-1* mutation is osmo-remediable and is rescued by an N-terminal truncated version of α-COP containing a truncated WD40 motif, but not by a version lacking the entire WD40 domain (Lee *et al.*, 1999, 2001).

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We also presented evidence that the *soo1-1* mutation causes defect(s) in the post-translational modification and assembly of yeast cell-wall proteins, which may contribute to the maintenance of cell wall integrity (Lee *et al*., 2001; Kim and Park, 2004; Kim *et al*., 2011).

In order to understand the function of the WD40 domain of α-COP in interacting with other subunits of the COPI coatomer, and thus its role in construction and/or maintenance of yeast cell walls, especially at elevated temperature, we generated various substitutions at G227 and deletions of the WD40 domain in α-COP, and performed a complementation test for the temperature-dependent osmo-sensitivity (TOS). Systematic mapping of interaction domains on α-COP was also performed to identify the regions required for interaction with other subunits of COPI vesicles, such as β-COP and ε-COP.

Materials and Methods

Strains and growth media

Saccharomyces cerevisiae strains LP0353 (*MATα ura3-52 lys2-801 soo1-1 bgs2 gal2*) (Lee *et al*., 1999), LP0353RS1 (*MATα ura3-52 lys2-801 bgs2 gal2*) (Lee *et al*., 1999), DSH (*MATα ura3-52 his4-519 ade1-100 soo1::LEU2,* pGAL*SOO1*), in which the expression of *SOO1* could be regulated by galactose, and EGY48 (*MATa LEU2::LexA*$_{op(x6)}$*-LEU2 ura3 his3 trp1* Gal$^+$) (Clontech, USA) were used in this study. The yeast strains were grown in YEPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose) or synthetic minimal medium, SD (0.67% Bacto-yeast nitrogen base without amino acids, 2% dextrose). D-Sorbitol was added at a concentration of 1.2 M as an osmotic stabilizer, if necessary.

DNA manipulation and strain construction

Escherichia coli strain DH5α was used for plasmid isolation, and PCRs using *Pfu* DNA polymerase, restriction enzyme digests and ligations were performed according to standard methods (Sambrook, 1989). For *in vitro* mutagenesis, an overlap extension PCR method (Ho *et al*., 1989) was used. In brief, two pairs of primers were used to direct synthesis of mutated DNA fragments; fragments 1 and 2 were amplified from *S. cerevisiae* genomic DNA using primers A and B, and C and D, respectively. Primer sequences were as follows: A, 5′-ATGAAGATGTTAACTAAATTTG-3′; B, 5′-GATTGTCTCCNNNAGTAGC-3′; C, 5′-GTCAC TNNNGGAGACAATC-3′; and D, 5′-AATGGTAAAGAG CCGC-3′. B and C primers contained the randomly substituted three nucleotides (GGC to NNN) for amino acid 227 of α-COP. The PCR products, which overlapped by 19 bp, were gel-purified and subsequently used for overlap extension PCR with primers A and D. The resulting PCR products were gel-purified and digested with *Xba*I and *BstE*II restriction endonucleases. The digested fragments were used to replace the corresponding region within the wild-type *SOO1* gene of YCp*SOO1* (Lee *et al*., 1999). The mutant alleles generated were identified by nucleotide sequencing of the PCR products cloned. The DSH strain was obtained from tetrad-dissect of asci from the diploid FL82*ΔSOO1* (*MATa/MATα ura3-53/ura3-52 his4-519/HIS4 trp1-289/TRP1 ade1-100/ADE1 leu2-3/LEU2 ΔSOO1/SOO1*) which had been transformed with pGAL*SOO1* (YCplac33 had the *GAL1* promoter and *SOO1* allele). A pADH plasmid equipped with the *ADH1* promoter on pRS313 was used for expression of the mutant *soo1-1* alleles in DSH. For yeast two-hybrid analysis, the LexA two-hybrid system was used (Estojak *et al*., 1995; Golemis *et al*., 1996). Yeast reporter strain EGY48, the bait plasmid pLexA, and the prey plasmid pB42AD were obtained from Clontech. Prey fusions were under the control of the *GAL1* promoter. Fusion constructs were created by ligating PCR products, which had been prepared with custom primers, into the vectors above, generating in-frame fusions with LexA (bait

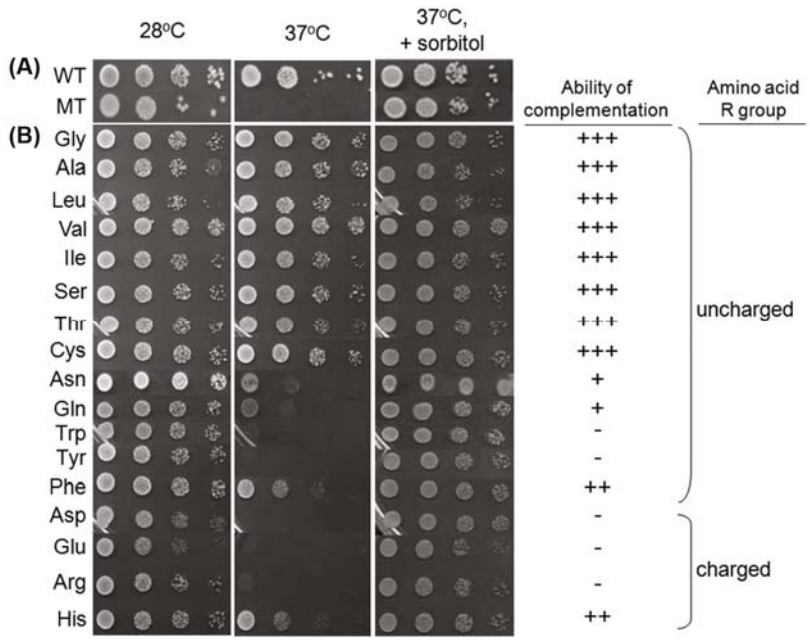

Fig. 1. Complementation test of the mutant alleles in LP0353. (A) Observation of temperature-dependent osmo-sensitive phenotype in wild-type LP0353RS1 and mutant LP0353 at permissive (28°C) and non-permissive (37°C) temperatures. Cell suspensions (2.5×10^5 cells/μl) were prepared and 4 μl of the undiluted and three different 10-fold dilutions thereof were dotted on YEPD with or without 1.2 M sorbitol, respectively. The growth was followed for 3 successive days. (B) Complementation test of the mutant alleles generated by the site-directed mutagenesis at position 227 of Soo1p in LP0353. Each transformant was dotted on selective plates (SC-URA) with or without 1.2 M sorbitol, respectively.

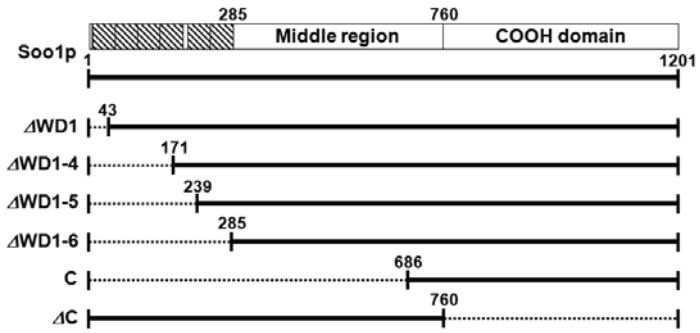

Fig. 2. Schematic representation of the serially deleted Soo1p. Wild-type Soo1p/Ret1p/α-COP has 1201 amino acids and six WD40 motifs in N-terminal WD40 domain are indicated by dotted boxes. The dotted line indicates the deleted region of Soo1p; ΔWD1, deletion of the first of six WD40 motifs in its N-terminus; ΔWD1–4, deletion of the first to the fourth WD40 motifs; ΔWD1–5, deletion of the first to the fifth WD40 motifs; ΔWD1-6, deletion of the first to the sixth WD40 motifs; C, deletion of amino acids 1–685; ΔC, deletion of amino acids 761–1201.

vector) or 'acid blob B42' (prey vector). For full-length fusion of the COPs, chimeras started at the first ATG codon of the open reading frames. For truncations, the positions of amino acids on the COPs are depicted in Fig. 2.

Two-hybrid assay

All methods were carried out according to Clontech yeast protocols. Reporter strain EGY48, harboring the *LacZ* reporter plasmid pSH18-34 (*URA3*; see Golemis *et al.*, 1996), was co-transformed with a bait and a prey fusion construct, and transformants were selected on -HIS, -TRP, -URA plates. To assess interaction between bait and prey, an independent transformant was streaked onto plates lacking histidine, tryptophan and uracil, containing 2% galactose, 1% raffinose, BU salt and X-Gal, and incubated at 30°C for 3 days. β-Galactosidase activity was tested on liquid with *o*-nitrophenyl-β-D-galactopyranose (ONPG) as substrate. Each experiment was repeated at least three times.

Western blot analysis of cell-wall proteins

Preparation and western analysis of the cell-wall proteins were performed by the previous methods (Lee *et al.*, 2002; Kim and Park, 2004). Briefly, cells grown at permissive or non-permissive temperature until the OD_{600}=1.0 were harvested by centrifugation, washed, and treated with glass beads in a Beadbeater (Biospec Products, USA). After removal of the glass beads, the cell walls were pelleted, washed 5 times with 5 M LiCl, and suspended in 0.5 ml of 50 mM Tris-HCl (pH 8.0) containing 2% SDS, 100 mM EDTA, and 40 mM dithiothreitol and then heated at 100°C for 10 min to harvest SDS-soluble cell-wall proteins. For the preparation of the covalently linked cell-wall proteins, the pelleted cell-wall fractions after SDS-extraction were washed five times with 1 mM phenylmethylsulfonyl fluoride and once with 50 mM Tris-HCl buffer (pH 8.0), and then digested with 20 units/ml of Quantazme (Qbiogene, Canada) in 0.2 ml of reaction buffer (67 mM KH_2PO_4; pH 7.5 and 120 mM β-mercaptoethanol) at 30°C for 6 h. The reaction mixture containing solubilized cell-wall proteins was centrifuged at 15,000×g for 10 min, and the supernatant was used as covalenly linked cell-wall proteins. For the analysis of cell-wall proteins, cell-wall protein samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech Co., USA) in a solution of 25 mM Tris, 192 mM glycine, 20% methanol, and 0.05% SDS using Semiphore transfer unit (Amersham Pharmacia Biotech Co., USA). The electroblotted membrane was saturated with blocking buffer (0.02% Tween 20 and 5% skim milk in TBST) for 1 h. Primary antibodies (mouse anti-enolase and mouse anti-Gas1p) were probed in membranes and developed with anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Amersham Phamacia Biotech Co.). Immunobloting was enhanced using the ECL labeling and detection kit (Amersham Phamacia Biotech Co.) as described in the manufacturer's manual. For the generation of primary antibodies, a fusion protein containing N-terminal glutathione S-transferase (GST) tag and amino acid residues 1-249 of Gas1p or 251-437 of enolase was expressed in *E. coli* and purified by GST column. Mice were subsequently immunized with the purified fusion protein, and the antiserum was obtained as previously reported (Lee *et al.*, 2002; Kim and Park, 2004).

Results and Discussion

The nature of the amino acid at position 227 affects the function of α-COP

Previously we reported that a G227D mutation in the fifth WD40 motif of *soo1-1/ret1-1* is responsible for TOS at 37°C (Lee *et al.*, 1999, 2001). We also reported that the *soo1-1* mutation impairs the glycosylation of cell wall proteins, which is crucial for the maintenance of cell wall integrity, by mislocalization of ER resident proteins (Lee *et al.*, 2002; Kim and Park, 2004; Kim *et al.*, 2011). To test the effect of substitutions at amino acid 227 of α-COP on the maintenance of yeast cell-wall rigidity, mutant alleles for substitution of G227 to other amino acids were generated by site-directed mutagenesis, introduced into LP0353, the *soo1-1* mutant, and tested for the ability to complement the TOS phenotype. As shown in Fig. 1, the ability to complement the TOS phenotype of the mutant alleles seemed to be dependent on the nature of amino acid 227. For example, amino acids that revealed complete or partial complementing activity had an uncharged non-polar R group, such as Ala, Leu, Ile, and Val, or an uncharged polar R group, such as Gly, Ser, Thr, Cys, Gln, and Asn. On the other hand, amino acids that revealed no complementing activity had a charged R group, such as Asp, Glu, and Arg, or an uncharged aromatic R group, such as Trp and Tyr, except for Phe. Interestingly, a positively charged amino acid, histidine,

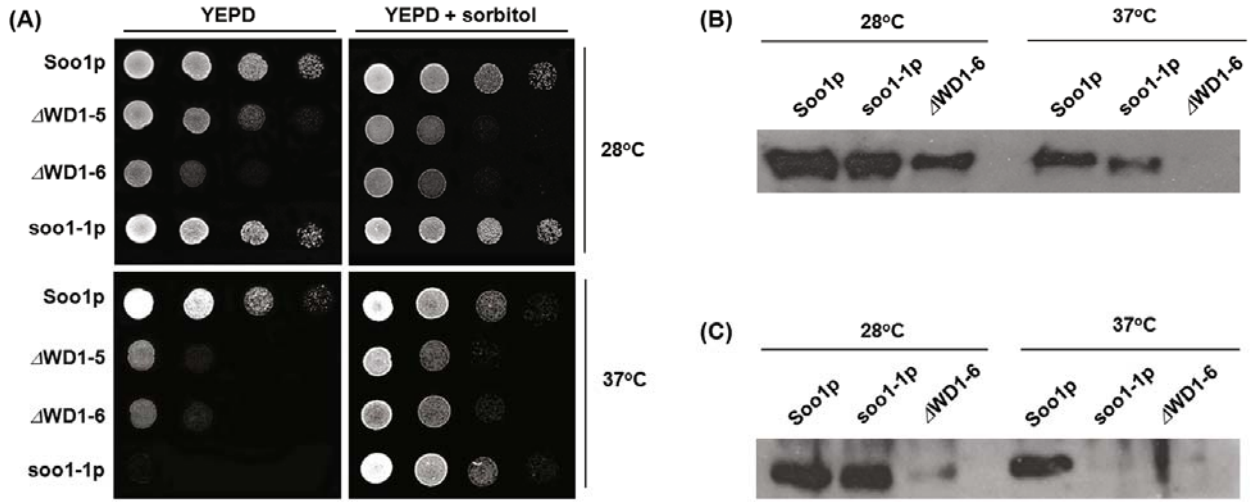

Fig. 3. Effect of mutations in the WD40 domain of α-COP on cell growth and cell wall proteins. (A) Growth phenotype of strains carrying serially deleted Soo1p in DSH. pGAL*SOO1* was removed from DSH in media containing 5-fluoroorotic acid (5-FOA) after transforming pADH with the *soo1* mutant allele. DSH carrying pADH, which has each allele, was spotted on media at the temperatures indicated. (B) and (C) Western blot analysis of cell wall proteins. Twenty microgram of proteins were loaded into each lane and separated on 10% SDS-PAGE. After transfer of the separated proteins to a PVDF membrane, each sample was analyzed with anti-Gas1p antibodies (B) or anti-enolase antibodies (C), respectively.

which can be uncharged depending on its local environment (Berg *et al.*, 1995) showed partial complementing activity. It is also noteworthy that the pK_a values of most of the amino acids without complementing activity were highly acidic (Asp and Glu, 4.4) or highly alkaline (Tyr, 10.0; Arg, 12.0).

Many regulatory proteins have a WD40 domain, which contains WD40 motifs that mediate protein–protein interactions. The WD40 motif comprises a 44~60-residue sequence, and typically contains the GH dipeptide 11~24 residues from its N-terminus and the WD dipeptide at the C-terminus. The WD40 motif forms four antiparallel β-strands (D, A, B, and C) producing sheets (Wall *et al.*, 1995), which in turn fold into a propeller-like structure. A large or charged residue in the conserved GH position possibly interferes with hydrophobic interactions and has the potential to disrupt the β-propeller (Wall *et al.*, 1995). The conserved Ser226 immediately adjacent to the *soo1-1* mutation contributes to the stability of the propeller blades (Schröder-Kohne *et al.*, 1998). However, it was noted that although the exception of Phe needs further analysis, uncharged amino acids with a bulky aromatic group at amino acid 227 showed no complementing activity.

Our results indicate that the *soo1-1/ret1-1* mutation might give rise to an impaired β-propeller structure. As previously reported (Holm *et al.*, 2001), it was suggested that not only charged amino acids but also large aromatic acids at position 227 might affect the intracellular function of Soo1p/α-COP by interfering with the formation of the β-propeller and thus protein–protein interaction of α-COP. These mutation, therefore, might lead to impairment of functional COPI vesicle formation especially at the elevated temperature (37°C).

Mutations in the WD40 domain of α-COP affect growth and assembly of cell-wall proteins

The WD40 domain of α-COP is dispensable for yeast cell viability, but a truncated version of α-COP, lacking the entire WD40 domain, supports yeast cell viability up to 34°C, but not at 37°C (Eugster *et al.*, 2000). However, the effect of truncation of the N-terminal WD40 domain on the TOS phenotype has not been characterized yet, and our previous results suggest a possible role of the WD40 motif in cell wall integrity (Lee *et al.*, 2001). To address this question, serial deletions of the WD40 motifs were constructed (Fig. 2) and introduced into a new strain, DSH, which has no chromosomal copy of *SOO1* but an episomal vector, pGAL*SOO1*, containing one copy of *SOO1* under the control of the regulatable *GAL1* promoter. Under culture conditions for curing the episomal *SOO1* and expressing the truncated *SOO1* under the *ADH1* promoter, the ability to complement the TOS phenotype was tested.

As shown in Fig. 3A, DSH (Soo1p) showed almost the same growth pattern at both temperatures, regardless of the presence or absence of an osmotic stabilizer. The truncated versions of *SOO1*/α-COP, namely, ΔWD1-5 and ΔWD1-6, were able to support cell growth at both temperatures, but showed a reduced growth rate at both temperatures, even in the presence of osmotic stabilizer. It is also noteworthy that the growth rate of the strain with α-COP lacking the entire WD40 domain (ΔWD1-6) was lower than that with α-COP containing the last WD40 motif only (ΔWD1-5) at 28°C without sorbitol. These results indicate the involvement of the WD40 motif of α-COP for yeast cell growth and cell wall biogenesis. In order to test the effect of the WD40 domain of α-COP on cell wall biogenesis such as assembly of cell-wall proteins, western analysis of the SDS-soluble cell-wall protein, enolase (Eugster *et al.*, 2004; Lopez-Villar *et al.*, 2006), and covalently linked cell-wall protein, Gas1p (De Sampaio *et al.*, 1999), were performed. As shown in Figs. 3B and 3C, reduction and/or almost complete loss of the Gas1p and enolase were evident in the cell-wall protein preparations from the strain with

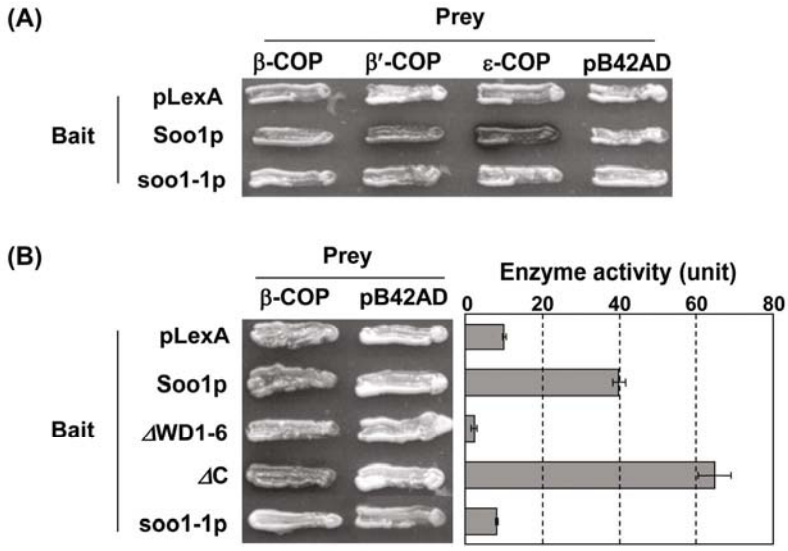

Fig. 4. Interaction of soo1-1p with its interactor, β-, ε-, and β′-COP. (A) Wild type or *soo1-1* mutant α-COP was expressed in the bait vector (pLexA) and tested for interaction with full-length β-, β′-, and ε-COP in the prey vector (pB42AD), respectively. (B) Interaction between truncated α-COP and β-COP. A β-galactosidase assay (right panel) was carried out. Results for β-galactosidase activity are the mean of assays carried out in triplicate. Error bars represent the standard error of the mean.

soo1-1 mutation as well as the strain with α-COP lacking the entire WD40 domain (ΔWD1-6).

It was also noted that expression of the *soo1-1* mutant allele in DSH showed that the TOS phenotype was suppressed by sorbitol at a non-permissive temperature, as expected, but the reduced growth rate exerted by truncation of the WD40 motifs was not suppressed by sorbitol (Fig. 3A). These results indicate that deletion and point mutations in the WD40 domain did not exert the same effect on the intracellular function of α-COP, and further suggest that the mutant form of the WD40 domain in soo1-1p/ret1-1p might act as a negative *cis*-acting element in the interaction of α-COP with other coatomers.

soo1-1/ret1-1 mutant α-COP does not interact with β- and ε-COP but interacts with β′-COP

In order to investigate the effect of the *soo1-1/ret1-1* mutation on interactions of α-COP with β-, β′-, and ε-COP, respectively, interactions of full-length coatomer subunits were analyzed by a yeast two-hybrid system. In contrast to wild-type Soo1p/α-COP, which showed interaction with β-COP, β′-COP, and ε-COP (Eugster *et al.*, 2000), soo1-1p/ret1-1p did not show interaction with β-COP or ε-COP (Fig. 4A). These results were comparable to the previous report, which showed the interaction of an α-COP truncation lacking the entire WD40 domain with full-length ε-COP and the requirement of the C-terminal ~170 amino acids of α-COP for direct interaction with ε-COP in the two-hybrid and crystal structure analysis (Eugster *et al.*, 2000; Hsia and Hoelz, 2010). Here we report the novel finding that although the WD40 domain was dispensable for interaction of α-COP with ε-COP, the G227D mutation present in the WD40 domain impairs the interaction of α-COP with ε-COP. In contrast to the wild-type Soo1p, no interaction between soo1-1p and β-COP indicates abolition of the α- and β-COP interaction by the G227D mutation, and thus the possible involvement of the N-terminal WD40 domain in α- and β-COP interaction. On the contrary, the interaction between soo1-1p and β′-COP indicated no effect of the G227D mutation of α-COP on its interaction with β′-COP. Yeast two hybrid analysis reveals that three separable regions of full-length α-COP that can interact with β′-COP in a manner independent of the WD40 domain of β′-COP (Eugster *et al.*, 2000). Domain analysis performed with limited proteolysis to define domain boundaries and identify protein interactions in the αβ′ε-COP sub-complex also reveals that the interaction between α-COP and β′-COP requires an almost full-length β′-COP (residues 1-798) and a central α-solenoid domain of α-COP (residues 638-813) (Lee and Goldberg, 2010). Therefore, the G227D mutation of α-COP alone does not affect the interaction between soo1-1p and β′-COP; instead, this interaction seems to be mediated by multiple domains in both COP proteins. Our results shown here suggest the possible involvement of the WD40 domain in the interaction of α-COP with β-COP and ε-COP, respectively.

Interaction of α-COP with β-COP relies on the WD40 domain

To gain further insight into the role of the α-COP WD40 domain in coatomer assembly, interactions of the truncated versions of α-COP with β-COP were analyzed by the two-hybrid assay. Fig. 4B shows that an α-COP truncation lacking the entire WD40 domain did not interact with full-length β-COP, but an α-COP truncation lacking the C-terminal domain was able to interact with full-length β-COP.

Although a previous report showed that the interaction of α-COP with β-COP is mediated by the C-terminal region of β-COP (Eugster *et al.*, 2000), the α-COP region responsible for α- and β-COP interaction had not yet been identified. Further analysis should be done to identify the precise α-COP domain responsible for interaction with β-COP, however, our results clearly show the requirement of the WD40 domain of α-COP for interaction with β-COP.

Alteration of the α-COP domain affects interaction with ε-COP

A unique α-COP mutation located at the C-terminus, *ret1-3*,

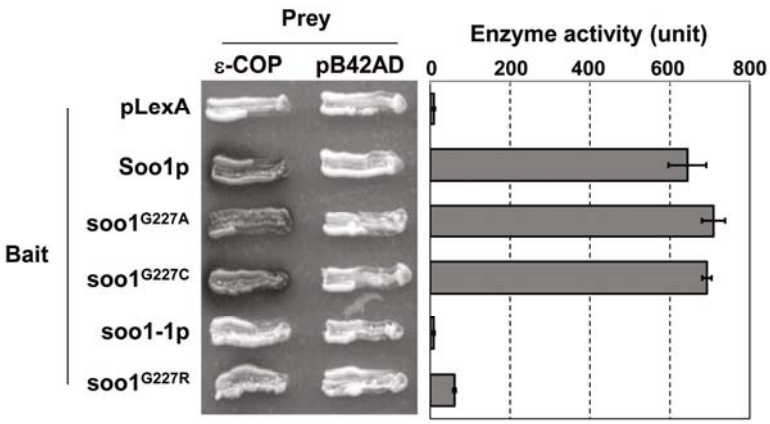

Fig. 5. Effect of substituted amino acid 227 in α-COP on interaction between Soo1p and ε-COP. Wild-type and substituted versions of α-COP were expressed in the bait vector, respectively and tested for interaction with ε-COP expressed in the prey vector. Left panel, X-gal plate test; right panel, β-galactosidase assay.

causes structural alterations to coatomer, α-COP degradation and forward transport defects at restrictive temperatures; all these phenotypes can be rescued by over-expression of ε-COP (Duden *et al*., 1998). Truncation of the last ~170 residues of α-COP abolishes binding to ε-COP without affecting interaction with β′-COP, but α-COP truncation lacking the entire WD40 domain imposes no adverse effect on interaction with ε-COP. Thus, the C-terminal ~170 amino acids of α-COP are required for direct interaction with ε-COP (Eugster *et al*., 2000; Hsia and Hoelz, 2010). Although the WD40 domain of α-COP is known to be dispensable for interaction with ε-COP, Fig. 4 shows the severe reduction in soo1-1p interaction with ε-COP. Therefore, the effect of other amino acid changes in position 227 of α-COP on interaction with ε-COP was analyzed. The effect of an uncharged R-group amino acid such as Ala and Cys was opposite to that for charged groups such as Asp and Arg (Fig. 5). To gain further insight into the function of the WD40 domain, α-COP truncations were tested against full-length ε-COP (Fig. 6). Interestingly, the C-terminal half region alone showed almost the same level of interaction with ε-COP as that of full-length α-COP, but the α-COP truncation lacking the C-terminal region did not interact with ε-COP, as previously reported (Eugster *et al*., 2000). However, all versions of α-COP with a WD40 truncation tested (ΔWD1, ΔWD1-4, ΔWD1-5, and ΔWD1-6) showed severely reduced interaction with ε-COP. Although the C-terminal of α-COP is responsible for its interaction with ε-COP, the results described here indicate that the WD40 domain of α-COP is also involved in mediating interaction between these two COP proteins; amino acid substitutions in the WD40 domain are just as deleterious as deletions in the WD40 domain (Figs. 5 and 6) with regard to α-COP and ε-COP interactions. Although further structural analyses are required, these results suggest that modifications in the WD40 domain of α-COP may cause conformational changes in the three-dimensional structure of α-COP, and thus, they affect the interaction between α-COP and ε-COP. Taken together, it can be postulated that, although it is dispensable, the structurally altered WD40 domain might act as a negative *cis*-acting element, which could severely impair the interaction of α-COP with ε-COP. The lack of effect of the *soo1-1*/*ret1-1* mutation on α-COP interaction with β′-COP could be explained by the hypothesis that α-COP tightly interacts with β′-COP over three independent binding sites (Eugster *et al*., 2000).

In summary, our results indicate that the interaction of α-COP with ε-COP or β-COP relies on the WD40 domain of α-COP, although biochemical assays such as co-immunoprecipitation are needed to confirm these findings. In addition, it can be postulated that the inability of *soo1-1* mutant cells to grow at non-permissive temperatures may be due not only to the failure of soo1-1p interaction with ε-COP and β-COP, but also to the failure of retrograde

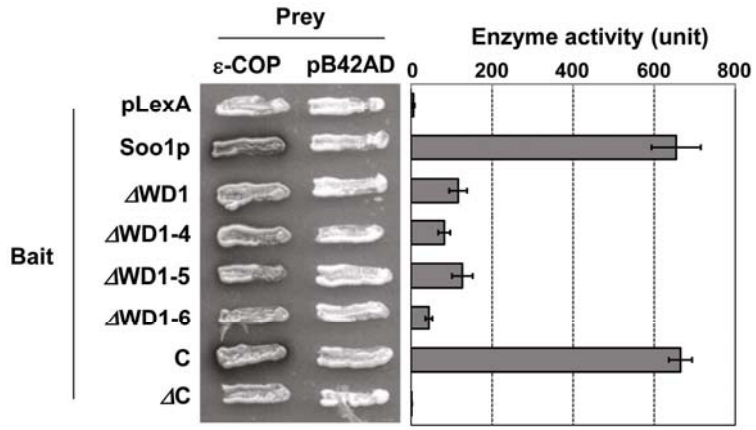

Fig. 6. Role of the WD40 domain of α-COP in interaction with ε-COP. Wild-type and truncated versions of α-COP were expressed in the bait vector, respectively and tested for interaction with ε-COP expressed in the prey vector. Left panel, X-gal plate test; right panel, β-galactosidase assay.

transport of KKXX-tagged ER-resident proteins, which are essential for post-translational processing of proteins for cell wall biogenesis. Indeed, our recent studies indicate that Golgi-to-ER retrograde transport of the KKXX-tagged ER-resident proteins, Wbp1 and Pmt2p, responsible for protein glycosylation (Eugster *et al.*, 2000; Beck *et al.*, 2009) is blocked by the *soo1-1* mutation at 37°C (Kim *et al.*, 2011).

Acknowledgements

This work supported by a Korea Research Foundation Grant (KRF-2001-DP0512).

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