The α- and β'-COP WD40 Domains Mediate Cargo-selective Interactions with Distinct Di-lysine Motifs

Anne Eugster,* Gabriella Frigerio,* Martin Dale, and Rainer Duden†

Department of Clinical Biochemistry, Cambridge Institute for Medical Research, University of Cambridge, Cambridge CB2 2XY, United Kingdom

Coatomer is required for the retrieval of proteins from an early Golgi compartment back to the endoplasmic reticulum. The WD40 domain of α-COP is required for the recruitment of KKTN-tagged proteins into coatomer-coated vesicles. However, lack of the domain has only minor effects on growth in yeast. Here, we show that the WD40 domain of β'-COP is required for the recycling of the KTKLL-tagged Golgi protein Emp47p. The protein is degraded more rapidly in cells with a point mutation in the WD40 domain of β'-COP (*sec27-95*) or in cells lacking the domain altogether, whereas a point mutation in the Clathrin Heavy Chain Repeat (*sec27-1*) does not affect the turnover of Emp47p. Lack of the WD40 domain of β'-COP has only minor effects on growth of yeast cells; however, absence of both WD40 domains of α- and β'-COP is lethal. Two hybrid studies together with our analysis of the maturation of KKTN-tagged invertase and the turnover of Emp47p in α- and β'-COP mutants suggest that the two WD40 domains of α- and β'-COP bind distinct but overlapping sets of di-lysine signals and hence both contribute to recycling of proteins with di-lysine signals.

INTRODUCTION

COP I coat proteins mediate an essential and conserved retrieval pathway that continually recycles several classes of proteins, and lipids, from the *cis*-Golgi back to the endoplasmic reticulum (ER) (Letourneur *et al.*, 1994; Gaynor *et al.*, 1998; Lanoix *et al.*, 1999). Additionally, the COP I coat functions in retrograde transport and perhaps also in antero-grade transport, within the Golgi complex (Orci *et al.*, 1997; Pelham and Rothman, 2000). The minimal machinery for the budding of COP I-coated vesicles consists of coatomer, a stable cytosolic complex comprising seven equimolar subunits, α-, β-, β'-, γ-, δ-, ε-, and ζ-COP, and the small GTPase ARF in its GTP-bound form (Rothman and Wieland, 1996; Spang *et al.*, 1998). Binding of ARF-GTP to Golgi membranes leads to recruitment of coatomer, deformation of the membrane, and budding of COP I-coated vesicles. GTP hydrolysis by ARF is a prerequisite for vesicle uncoating (Rothman and Wieland, 1996). The GTP/GDP cycle of ARF proteins is regulated by guanine nucleotide exchange factors and GTPase-activating proteins (GAPs) (Donaldson and Jackson, 2000).

Cargo transported in COP I vesicles in mammalian cells and yeast includes type I membrane proteins harboring a di-lysine trafficking signal, which functions only when the lysines are either in -3 and -4, or -3 and -5 positions from the carboxy terminus. This di-lysine motif is necessary and sufficient to target reporter proteins into the retrograde pathway in vivo, and coatomer can directly interact with such motifs in vitro (Jackson *et al.*, 1993; Gaynor *et al.*, 1994; Cosson and Letourneur, 1994; Schröder-Kohne *et al.*, 1998). Using reporter proteins tagged with variants of di-lysine

* These authors contributed equally to this study.
† Corresponding author. E-mail address: rd217@cam.ac.uk.

motifs, it has been previously demonstrated clearly that small changes in the local sequence context of the lysines can dramatically alter the signal strength of the di-lysine motif and thus generate a broad spectrum of trafficking phenotypes (Zerangue *et al.*, 2001).

In this study, we analyze in detail interactions of coatomer with variants of the di-lysine motifs as present on two well characterized proteins in yeast: Wbp1p, a subunit of the ER-localized oligosaccharyl transferase complex (Gaynor *et al.*, 1994); and Emp47p, a protein that achieves its steady-state Golgi localization by continuous recycling between the endoplasmic reticulum (ER) and the Golgi (Schröder *et al.*, 1995; Schröder-Kohne *et al.*, 1998). Emp47 and its close homologue Emp46p have been proposed to function as cargo receptors at the ER exit site (Sato and Nakano, 2002, 2003). Wbp1p harbors a classical di-lysine motif, KKTN, and Emp47p presents a variant di-lysine motif, KTKLL (Gaynor *et al.*, 1994; Schröder *et al.*, 1995). A genetic selection for yeast mutants with specific defects in trafficking dependent on the di-lysine KKTN motif, as present on Wbp1p, yielded mutations in α-, γ-, δ-, and ζ-COP, which provided strong evidence for a function of COP I in Golgi-to-ER retrieval (Letourneur *et al.*, 1994). In particular, *ret1-1*, an α-COP mutant, displays a strong in vivo KKTN-retrieval defect even at permissive temperature, and coatomer from *ret1-1* cells has lost the ability to interact with the KKTN-motif in vitro. Anterograde traffic is virtually unaffected in this mutant even upon shift to restrictive temperature (Letourneur *et al.*, 1994). Significantly, the point mutations in four different alleles of *ret1* identified in the selection, including *ret1-1*, cluster within 85 residues in or close to an amino-terminal domain containing WD40 repeats, providing strong genetic evidence for a role of this domain of α-COP in KKTN-binding (Letourneur *et al.*, 1994; Schröder-Kohne *et al.*, 1998). In vitro, an α-, β'-, ε-COP subcomplex strongly binds to the KKTN motif (Lowe and Kreis, 1995). Consistent with these data an artificial, very strong di-lysine signal, KKYL, isolated in a systematic screen for ER trafficking signals in

Table 1. Yeast strains used

Strain	Genotype	Reference
EGY 48	*MATα, his3, trp1, ura3, leu2*::*pLEU2-lexAop3*	Gyuris *et al.*, 1993
EGY191	*MATα, his3, trp1, ura3, leu2*::*LEU2-LexAop1*	Gyuris *et al.*, 1993
PC70	*MATα, ret1-1, ura3, trp1, leu2*	Letourneur *et al.*, 1994
WPY153	*MATα, sec27-95, ura3, his3, leu2*	Prinz *et al.*, 2000
RDY146	*MATα, sec27-1, ura3, trp1, leu2*	Duden *et al.*, 1994
YPH500	*MATα, ura3, lys2, ade2, trp1, his3, leu2*	Sikorski and Hieter, 1989
YAE2-1c	*MATa, ura3, lys2, ade2, trp1, his3, leu2, ret1Δ*::*HIS3 + pCEN*::*RET1(CEN, URA3)*	Eugster *et al.*, 2000
YAE6	*MATa, ura3, lys2, ade2, trp1, his3, leu2, ret1Δ*::*HIS3 + pCEN*::*ret1Δ1-285 (CEN, LEU2)*	Eugster *et al.*, 2000
YAE7	*MATa, ura3, lys2, ade2, trp1, his3, leu2, ret1Δ*::*HIS3 + pCEN*::*RET1 (CEN, LEU2)*	Eugster *et al.*, 2000
YAE8-13c	*MATα, ura3, lys2, ade2, trp1, his3, leu2, sec27Δ*::*LEU2 + pCEN*::*SEC27 (CEN, URA3)*	This study
YAE9-3c	*MATα, ura3, lys2, ade2, trp1, his3, leu2, ret1Δ*::*HI3, sec27Δ*::*LEU2 + pCEN*::*ret1Δ1-285 (CEN, LEU2) + pCEN*::*SEC27 (CEN, URA3)*	This study
YAE10	*MATα, ura3, lys2, ade2, trp1, his3, leu2, sec27Δ*::*LEU2 + pCEN*::*sec27Δ1-285 (CEN, TRP1)*	This study
YAE11	*MATα, ura3, lys2, ade2, trp1, his3, leu2, sec27Δ*::*LEU2 + pCEN*::*SEC27 (CEN, TRP1)*	This study
YAE13	*MATa/α, ura3, lys2, ade2, trp1, his3, leu2, RET1/ret1Δ*::*HI3, SEC27/sec27Δ*::*LEU2 + pCEN*::*ret1Δ1-285 (CEN, LEU2) + pCEN*::*sec27Δ1-285 (CEN, TRP1)*	This study

mammalian cells was shown to bind to α-COP in the yeast two-hybrid assay (Zerangue *et al.*, 2001).

These data suggested that the α-COP subunit of coatomer is the receptor for di-lysine motifs in both mammals and yeast. Intriguingly, however, it was found that the recycling of Emp47p, which harbors the variant motif KTKLL, is unaffected in all α-COP mutants that strongly mislocalize KKTN-tagged proteins. Thus, α-COP cannot be the only receptor for di-lysine tagged COP I cargo proteins in yeast (Schröder *et al.*, 1995; Schröder-Kohne *et al.*, 1998). Both photo-cross-linking data (Harter *et al.*, 1996; Harter and Wieland, 1998) and in vitro binding data (Wu *et al.*, 2000) have suggested that a binding site for di-lysine motifs is present on mammalian γ-COP. An equivalent binding site has so far not been characterized in the yeast γ-COP orthologue. Furthermore, it is unknown which subunit in coatomer is responsible for binding variant di-lysine motifs, e.g., KTKLL. Thus, the exact nature of the molecular interactions that lead to sorting of cargo proteins harboring di-lysine type motifs into COP I vesicles is still controversial.

Both α- and β'-COP harbor conserved WD40 domains of ~285 residues in their amino-terminal regions. WD40 repeats are conserved sequence motifs of 44–60 residues with a GH dipeptide at the N terminus and a WD dipeptide near the C terminus of the repeat (Smith *et al.*, 1999). WD40 domains are widespread recognition modules thought to link partner proteins in intracellular networks of signaling and sorting. X-ray crystallographic data on WD40 domain-containing proteins, e.g., the β-subunit of heterotrimeric G proteins, and several other proteins (Wall *et al.*, 1995; Sondek *et al.*, 1996; Beisel *et al.*, 1999), suggest that WD40 domains generally fold into a β-propeller, a highly regular structure composed of β-strands and turns, capable of accommodating multiple ligands (Neer and Smith, 2000; ter Haar *et al.*, 2000; Holm *et al.*, 2001). The WD40 domains of α- and β'-COP comprise six and five WD40 repeats, respectively, and display high levels of sequence conservation across evolutionarily distant species (Duden *et al.*, 1994). Furthermore, the sequences of α- and β'-COP WD40 domains are highly related to each other, suggesting a possible overlap in their functions. In this study, we revisited the issue of the di-lysine signal-binding sites on coatomer. Our data suggest that the WD40 domains of α- and β'-COP bind distinct but overlapping sets of di-lysine signals, and that both domains contribute to target cargo proteins harboring di-lysine motifs into COP I vesicles in vivo.

MATERIALS AND METHODS

Yeast Strains and Media

Yeast strains used are listed in Table 1. Yeast media were either rich medium (YP), consisting of 1% Bacto Yeast extract, 2% Bacto Peptone, and either 2% glucose (YP-Glu) or 2% galactose/2% glycerol (YP-Gal/Gly), or minimal medium, containing 0.7% yeast nitrogen base (Difco, Detroit, MI) supplemented with appropriate amino acids (Sigma-Aldrich, St. Louis, MO) and the same carbon sources.

Fusion Constructs; Two-Hybrid Assays

The LexA two-hybrid system (Estojak *et al.*, 1995; Golemis *et al.*, 1996) was used as described previously (Eugster *et al.*, 2000). Yeast reporter strains EGY48 (Table 1), as well as the bait plasmids pEG202 and the prey plasmids pJG4-5 were used (Golemis *et al.*, 1996). Prey fusions were under the control of the *GAL1* promoter. Fusion constructs were created by ligating PCR products made using custom primers into vectors mentioned above, generating in frame fusions with LexA (in the bait vector) or the "acid blob B42' (in the prey vector)." Reporter yeast strain EGY48 (harboring *URA3* plasmid pSH18-34; Golemis *et al.*, 1996), was cotransformed with a bait and a prey fusion construct and transformants were selected on −HIS, −TRP, −URA plates. To assess growth on media lacking leucine, transformants were streaked onto plates lacking histidine, tryptophane, uracil, and leucine, containing 2% galactose/1% raffinose or 2% glucose, and incubated at 24 or 30°C for 3 or 4 d, respectively.

Immunoblotting, Radiolabeling, and Immunoprecipitation Antibodies

SDS-PAGE, immunoblot analysis by using enhanced chemiluminescence radiolabeling of cells, immunoprecipitation, and PhosphorImager quantitation were as described previously (Duden *et al.*, 1994, 1998; Eugster *et al.*, 2000). Rabbit antisera used were anti-coatomer, anti-α-COP, anti-β'-COP (Duden *et al.*, 1994); anti-γ-COP (Hosobuchi *et al.*, 1992); anti-ε-COP (Eugster *et al.*, 2000); anti-Sed5p (this study); and anti-Emp47p (Schröder-Kohne *et al.*, 1998). The α-COP fragment was specifically detected using an antiserum against a COOH-terminal α-COP peptide (residues 1155–1171 of Ret1p). Anti-carboxypeptidase Y (CPY), anti-invertase, anti-α-COP, and anti-Emp47p antisera were kindly provided by Drs. Randy Schekman (University of California, Berkeley, CA), Scott Emr (University of California, San Diego, CA), Pierre Cosson (University of Geneva, Switzerland), Francois Letourneur (Institut de Biologie et Chimie des Proteines, Lyon, France), and Stephan Schröder-Kohne (BioMedTec, Franken, Germany), respectively.

DNA Cloning, Sequencing, Computer Analysis, and Plasmids

Escherichia coli strain DH5α was used for plasmid isolation, and polymerase chain reaction (PCR) by using Vent DNA polymerase, restriction enzyme digests, and ligations were performed by standard methods. Database searches were performed using the BLAST and Ψ BLAST servers at National Institutes of Health. Multiple alignments were done with the program MEGALIGN by using the CLUSTAL method. Plasmids pEG1-KK and pEG1-QK encoding Inv.-Wbp1p fusion proteins harboring a KKTN or a nonfunctional QKTN motif (Gaynor *et al.*, 1994) were used in pulse-chase experiments (Duden *et al.*, 1998). For in vitro binding studies pGST-WBP1-KK
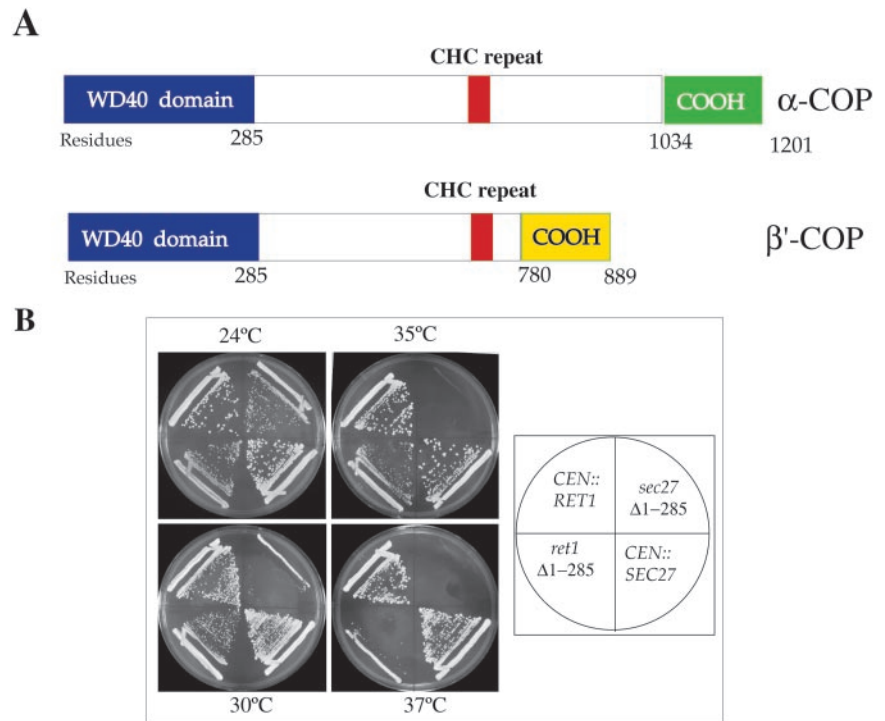
A

B

Figure 1. (A) Schematic of the domain structure of α- and β′-COP. In this study, we show that the WD40 domain of β′-COP (residues 1–285) is nonessential but required for selective interactions with a KTKLL motif, whereas the α-COP WD40 domain interacts with the "classical" KKTN-motif (Eugster *et al.*, 2000). A carboxy-terminal region of β′-COP, defined by *sec27-1* (a G688D mutation) is involved in maintaining α-COP stability and coatomer integrity (see text for details). (B) Growth phenotype of α- and β′-COP mutants harboring a deletion of the respective WD40 domain. Cells were streaked onto YEPD plates and grown for 3 d at the temperatures indicated. Strains were as indicated: wild-type control strains *CEN::RET1*, *CEN::SEC27* and mutant strains *CEN::ret1*Δ1-285 and *CEN::sec27*Δ1-285. Note that *sec27*Δ1-285 cells are strongly temperature sensitive.

and pGST-WBP1-SS (plasmids pFL67 and pFL68) encoding GST-fusions with the cytoplasmic tail of Wbp1p harboring either a KKTN or SSTN motif were used (Cosson and Letourneur, 1994), or KTKLL- or STSLL-GST fusions [i.e., harboring the wild-type Emp47p-tail (LIRQEIIKTKLL) or the corresponding double-serine mutant] (Schröder-Kohne *et al.*, 1998). pCEN::*sec27*Δ1-285 was created by introducing two PCR-amplified fragments: a *Bst*BI-*Bam*HI fragment containing the *SEC27* promoter and an artificial ATG, ending at the start ATG, and a *Bam*HI-*Apa*I fragment starting at residue 285 into *Bst*BI-*Apa*I cut pCEN::SEC27 (pRS314, *TRP*).

Strain Construction

A diploid *SEC27/sec27*Δ::LEU2 strain (Duden *et al.*, 1994) was transformed with the pRS316::*SEC27*, (*URA3*, *CEN*) plasmid and subsequently dissected, to yield the haploid strain YAE8-13c. To construct the strain expressing a truncated Sec27p lacking the WD40 domain as the only form of β′-COP at near endogenous level, YAE8-13c was transformed with pCEN::*sec27*Δ1-285 and pCEN::*SEC27* expressing the SEC27 protein under the control of the authentic *SEC27* promoter from a *TRP*, *CEN* plasmid. Ura−, Trp+, Leu+ colonies were selected on SD 5-fluoroorotic acid (5-FOA) plates, yielding the strains YAE10 and YAE11. In an attempt to construct a strain harboring deletions of the WD40 domains of both α- and β′-COP, the haploid strain YAE6 [*ret1*Δ ::HIS3 + pCEN::*ret1*Δ 1-285, (CEN, LEU2)] was crossed with the haploid strain YAE8-13c [*sec27*Δ ::LEU2 + pRS316::*SEC27*, (*CEN*, *URA3*)]. The diploid strain YAE9 *a*/α was sporulated and tetrads were dissected to yield YAE9-3c. YAE9-3c was transformed with pCEN::*sec27*Δ1-285 (CEN, TRP1) YAE9-3c and tested for viable Ura−, Trp+, Leu+, His+ colonies on SD 5-FOA plates. Alternatively, YAE6 [*ret1*Δ::HIS3 +pCEN::*ret1*Δ 1-285 (CEN, LEU2)] was crossed with the haploid strain YAE10 (*sec27*Δ::LEU2 + pCEN::*sec27*Δ 1-285 (*CEN*, *TRP1*)], the diploid strain YAE13 *a*/α was sporulated, 50 tetrads dissected, and screened for Leu+, His+, Trp+ colonies.

Cytosol Preparation; Gel Filtration; In Vitro Binding Experiments

Preparation of cytosol and Superose 6 gel filtration were as described previously (Duden *et al.*, 1998). GST-fusion proteins of Wbp1p-derived tails carrying a KKTN or SSTN motif, or of Emp47p-derived tails carrying a KTKLL or STSLL motif as described above, were expressed in *E. coli* and purified on glutathione-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ). For binding experiments, yeast spheroplasts were lysed at 100 OD$_{600}$/ml in HEPES-Triton buffer [50 mM HEPES, pH 7.3, 90 mM KCl, 0.5% Triton X-100, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. Cell lysates were centrifuged for 15 min at 20,000 × *g*. Supernatants, or in some experiments yeast cytosol prepared as described previously (Duden *et al.*, 1998) were incubated twice for 1 h at 4°C with beads containing GST only, followed by 2 h with beads harboring the fusion proteins. Beads were washed once in HEPES-

Triton buffer and five times in 50 mM HEPES, pH 7.3, and bound proteins eluted in SDS sample buffer and analyzed by immunoblot.

RESULTS

A novel β′-COP Mutant Lacking the WD40 Domain Is Viable, but Temperature-sensitive for Growth

α- and β′-COP across distant species harbor conserved N-terminal domains composed of WD40 repeats (Figure 1A, schematic representation). We previously demonstrated, by using a truncation mutant lacking this domain, that the WD40 domain of α-COP is not required for yeast cell viability but is essential for KKTN-dependent trafficking and binding to the KKTN peptide in vitro (Eugster *et al.*, 2000). Here, we investigate the function of the corresponding WD40 domain present on β′-COP. β′-COP in yeast is encoded by an essential gene, *SEC27* (Duden *et al.*, 1994). To test whether the WD40 domain on β′-COP is essential, we constructed a mutant, *sec27*Δ1-285, in which the only SEC27 protein present in the cells lacks this domain. The *sec27*Δ1-285 mutant is viable, but is temperature-sensitive (Figure 1B). Cells grow like wild-type cells at 24°C, but display severely reduced growth at 30°C. In comparison, the corresponding α-COP WD40 domain truncation mutant, *ret1*Δ1-285, grows well up to 35°C (Figure 1B; Eugster *et al.*, 2000). Thus, neither the α-COP nor the β′-COP WD40 domain is essential for yeast cell viability.

At 24°C all coatomer subunits are present in this β′-COP mutant at levels comparable to *ret1*Δ1-285 cells or congenic wild-type control cells (Figure 2). Coatomer from *sec27*Δ1-285 cells grown at 24°C fractionated identically to coatomer from wild-type cells upon gel filtration, and even after a shift for 3 h to restrictive temperature, 34°C, no differences were found between coatomer from *sec27*Δ1-285 cells and wild-type cells (our unpublished data). Thus, the temperature-sensitive growth defect dis-
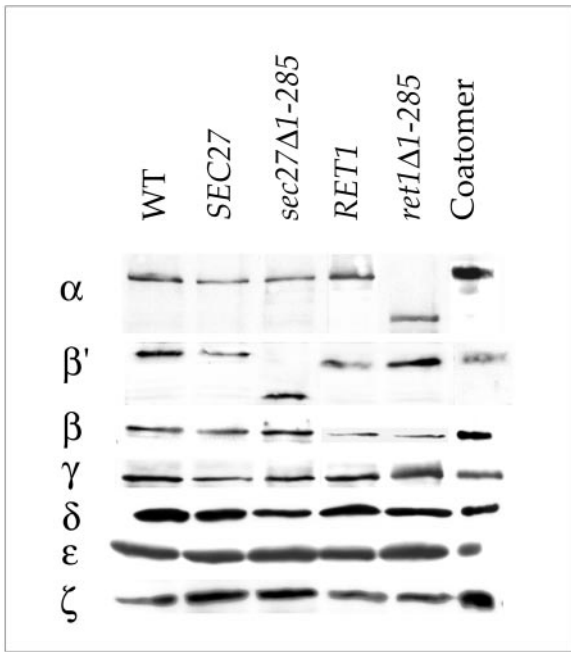
Figure 2. Levels of coatomer subunits in *sec27*Δ1-285 cells are normal. Whole cell extracts of *ret1*Δ1-285, *sec27*Δ1-285, control cells (*CEN::RET1* and *CEN::SEC27*), and wild-type cells (YPH500) grown at 24°C were analyzed by immunoblot on equal OD$_{600}$ equivalents of cells. Note the difference in molecular weight between wild-type and truncated α-COP or β′-COP. Further note that all other subunits are present in comparable amounts in all strains.

played by *sec27*Δ1-285 cells is not due to a gross defect in coatomer composition or structure.

At Least One WD40 Domain, on Either α- or β′-COP, Is Required for Yeast Cell Viability

Neither the α-COP WD40 domain nor the β′-COP WD40 domain is required for yeast viability individually. Next, we asked whether yeast cells that lack both WD40 domains are viable. Our data show that the inviability of a Δsec27::LEU2/Δret1::HIS3 double mutant cannot be rescued by simultaneous expression of truncated versions of α- and β′-COP that lack their WD40 domains (Figure 3). Expression of the truncated proteins was achieved using plasmids

pCEN::*sec27*Δ1-285 and pCEN::*ret1*Δ1-285, which can rescue the single deletion of the *SEC27* or *RET1* gene (Eugster *et al.*, 2000). We also tried to construct a double truncation mutant strain by genetic crosses, and again no viable double mutants were obtained (see MATERIALS AND METHODS). Thus, the presence of at least one WD40 domain in coatomer is essential for yeast cell viability. The synthetic lethality resulting from a simultaneous deletion of both WD40 domains prompted us to test whether the domains may overlap in their function.

Yeast Cells Harboring Point Mutations in β′-COP

To date only two β′-COP yeast mutants have been described, *sec27-1* (Duden *et al.*, 1994) and *sec27-95* (Prinz *et al.*, 2000). The *sec27-1* mutant was originally isolated in a screen for mutants with defects in mitotic spindle formation (Duden *et al.*, 1994). On shift to a restrictive temperature, *sec27-1* cells display a modest defect in KKTN-dependent trafficking and also mislocalize Emp47p, a KTKLL-motif bearing protein, to the vacuole (Letourneur *et al.*, 1994; Schröder-Kohne *et al.*, 1998). Coatomer in cytosol from temperature-shifted *sec27-1* cells shows a reduced ability to bind to KKTN-peptide in vitro (Letourneur *et al.*, 1994), but unlike with coatomer from *ret1-1* cells, binding to the KKTN-motif is not abolished completely. On shift to restrictive temperature the *sec27-1* mutant cells accumulate ER precursor forms of carboxypeptidase Y (p1-CPY) and α-factor, thus displaying also a modest anterograde transport defect (Duden *et al.*, 1994). The *sec27-95* mutant was identified in a visual screen for mutants that show defects in the structure of the cortical endoplasmic reticulum at restrictive temperature (Prinz *et al.*, 2000). Coatomer in cytosol from *sec27-95* mutant cells grown at a permissive temperature, 24°C, fractionates like wild-type coatomer upon gel filtration, and levels of all coatomer subunits are comparable to wild-type cells, suggesting that coatomer biosynthesis and stability are normal (our unpublished data). The trafficking phenotype in the *sec27-95* mutant has not been characterized so far and is described in this study.

In the hope that a molecular characterization of the mutations in *sec27-1* and *sec27-95* cells might help us to better understand domains on β′-COP, we undertook DNA sequencing of the *sec27* genomic regions in these mutants after PCR amplification. *sec27-1* harbors a point mutation in a carboxy-terminal region, changing glycine at position 688 to aspartic acid. The *sec27-95* mutation affects a residue in the amino-terminal WD40 domain, changing serine at position
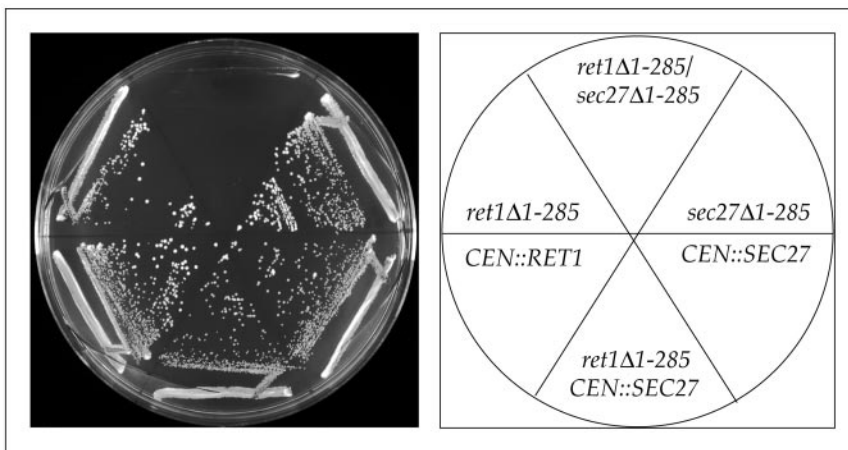

Figure 3. A strain lacking the WD40 domains of both α-COP and β′-COP is inviable. The mutant strains *ret1*Δ1-285 + pCEN::RET1 (*URA3*), *sec27*Δ1-285 +pCEN::SEC27 (*URA3*), *ret1*Δ::HIS3/sec27Δ::LEU2 +pCEN::ret1Δ1–285 +pCEN::sec27Δ1-285 +pCEN::SEC27 (*URA3*) and congenic control strains CEN::RET1 +pCEN::RET1 (*URA3*), CEN::SEC27 +pCEN: :SEC27 (*URA3*), and *ret1*Δ::HIS3/ sec27Δ::LEU2 +pCEN::ret1Δ1–285 +pCEN:: SEC27 +pCEN::SEC27 (*URA3*) were streaked out onto 5-FOA plates to induce loss of the URA3 plasmids, and incubated at 24°C for 4 d. Note that a *ret1*Δ1-285/*sec27*Δ1-285 strain is not viable without the pCEN::SEC27 plasmid.
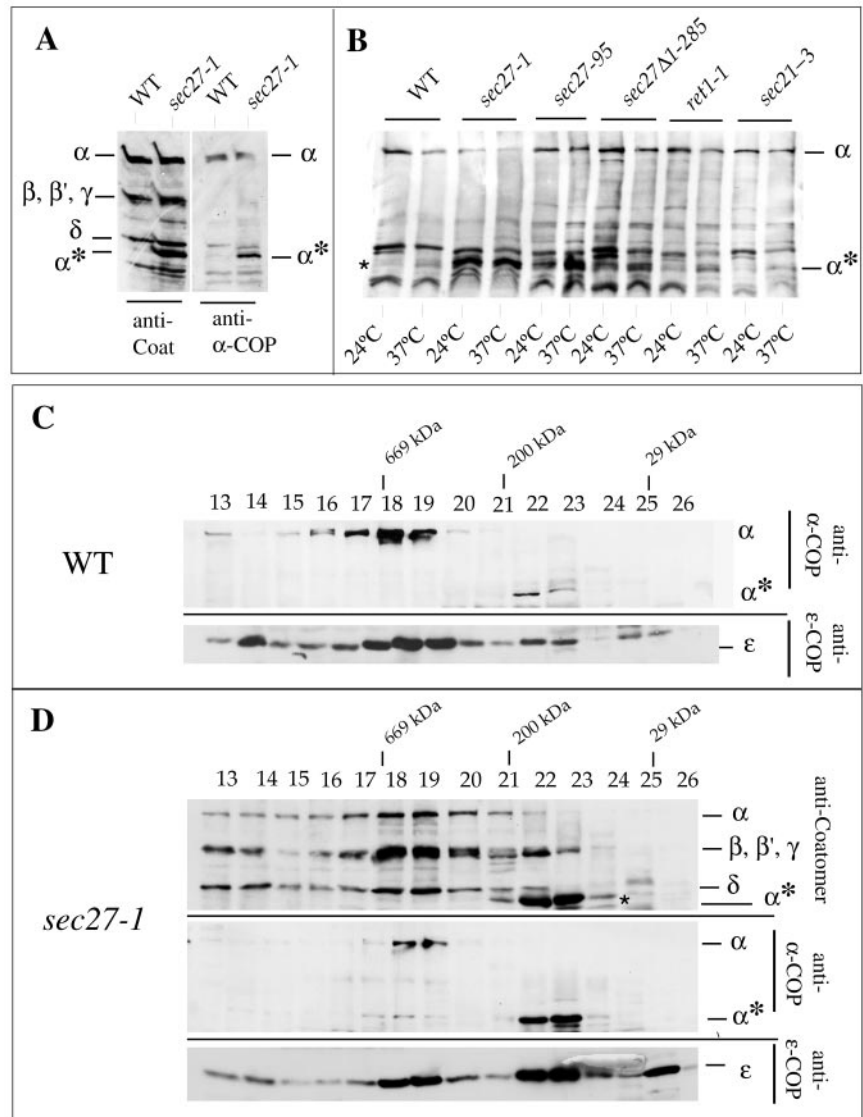
Figure 4. α-COP is destabilized in *sec27-1* cells. (A and B) Identification of a ~65-kDa α-COP fragment (α*) in *sec27-1* cells and other coatomer mutants as indicated, and in wild-type cells (WT). For details, see text. (C) Superose 6 gel filtration of cytosolic proteins from temperature-shifted wild-type cells (WT). α-COP species were identified with the α-COP peptide antiserum, and ε-COP with a specific rabbit antiserum. (D) Superose 6 gel filtration of cytosolic proteins from *sec27-1* cells. SDS-PAGE separated proteins of column fractions were probed with anti-coatomer antiserum to identify α-, β'-, β-, and γ-COP and the α-COP fragment (α*) (top). The antiserum against the carboxy-terminal α-COP peptide ("anti-α-COP") was used (middle); the bottom panel visualizes ε-COP. Note the reduced signal for intact α-COP in fractions 18 + 19 from *sec27-1* cells compared with wild type. Further note that a large fraction of α-COP immunoreactivity is present in fractions 22 + 23, corresponding to a partial complex of ~200 kDa in which ε-COP cofractionates. An additional peak of monomeric ε-COP is seen in fraction 26. Fraction numbers and positions of marker proteins are indicated: thyroglobulin (669,000), β-amylase (200,000), and carboanhydrase (29,000).

114 to tyrosine. In this study, we have used the two point mutants, as well as *sec27Δ1-285* cells, to analyze the functions of the amino- and carboxy-terminal domains of β'-COP.

α-COP Is Destabilized in the sec27-1 Mutant

Intriguingly, *sec27-1* cells display defects for both KKTN- and KTKLL-tagged proteins, but only upon a shift to restrictive temperature (Letourneur *et al.*, 1994; Schröder-Kohne *et al.*, 1998). Neither of these defects is apparent at permissive temperature. Therefore, to learn more about the nature of the defect in coatomer in *sec27-1* cells we shifted *sec27-1* cells, and wild-type cells as a control, for 2 h to 37°C and prepared cytosol. When immunoblotting cytosolic proteins with an antiserum against coatomer that recognizes all seven COPs (Duden *et al.*, 1994), we noticed a prominent additional band of ~65 kDa from *sec27-1* cells that was undetectable in wild-type extracts at the same exposure (Figure 4A). To identify this band, we immunoblotted the extracts with antisera against individual coatomer subunits. No difference between wild-type and *sec27-1* extracts was found with an-

tisera against β'-, β-, γ-, and δ-COP (our unpublished data) but the ~65-kDa band was recognized by an antiserum specific to a carboxy-terminal peptide of yeast α-COP (residues 1155–1171 of Ret1p) (Figure 4A). The additional band is thus an α-COP proteolytic fragment ("α*" in Figure 4) that roughly corresponds to the carboxy-terminal half of α-COP.

We next tested whether this α-COP fragment still associates with coatomer. On gel filtration of cytosol from temperature-shifted wild-type cells full-length α-COP cofractionated with all other COPs in fractions 18 + 19 that correspond to intact coatomer complex, as expected (Figure 4C). The ~65-kDa α-COP fragment was exclusively present in a partial complex of ~200 kDa, which also contained a peak of ε-COP (Figure 4, C and D). Although the ~65-kDa α-COP fragment was detectable from wild-type cells (Figure 4C), its abundance was dramatically increased in temperature-shifted *sec27-1* cells, concomitant with a decrease in full-length α-COP (Figure 4D). In wild-type cells, ε-COP was predominantly present in fractions 18 + 19, but in *sec27-1* cells ε-COP was mostly present in fractions 22 + 23 (i.e., in the partial ~200-kDa complex) and in fraction 26, as mono-

meric ε-COP (Figure 4D). Additionally, also β'-COP was detected in a peak in fractions 22 + 23 in *sec27-1* cells (Figure 4D; our unpublished results). Clearly, the structural integrity of coatomer is severely compromised in *sec27-1* cells upon shift to restrictive temperature, a phenomenon not observed in most other temperature-shifted coatomer mutant strains, including *sec27Δ1-285* cells (our unpublished data). However, this behavior is somewhat reminiscent of *ret1-3*, a strain harboring a point mutation (S1188F) in the carboxy-terminal region of α-COP, in which the stability of both α- and ε-COP, and thus of coatomer, is compromised at high temperature (Duden *et al.*, 1998; Eugster *et al.*, 2000).

Next, we analyzed whether the presence of the ~65-kDa α-COP fragment was specific to *sec27-1* cells. We prepared total cell lysates from wild-type cells and several coatomer mutants, *sec21-3* (γ), *ret1-1* (α), *ret1-3* (α), *sec27-1* (β'), *sec27-95* (β'), *ret2-1* (δ), *sec28Δ* (a strain lacking ε-COP), and *ret3-1* (ζ), either grown at 24°C or after a 2-h shift to 37°C. Total cell extracts were probed for presence of the ~65-kDa α-COP fragment by immunoblotting. At 37°C, the α-COP fragment, although most prominent in *sec27-1* cells, was detectable in all coatomer mutants to varying degrees, and a small amount even in wild-type cells (Figure 4B). This suggests that this α-COP fragment represents an intermediate in the normal cellular turnover of α-COP. Previously, we had noticed a peak of ε-COP in an identically sized partial complex of coatomer in yeast cells overexpressing α-COP (Eugster *et al.*, 2000). Using the anti-α-COP peptide antiserum, we report here that this partial coatomer complex contains clipped α-COP in addition to ε-COP (Figure 4D).

Significantly, in *sec27-1* cells the α-COP fragment was prominent even at 24°C. Thus, in this β'-COP mutant α-COP is more proteolytically susceptible, and thus coatomer less stable, than in all other coatomer mutants tested (Figure 5B; our unpublished data). This observation provides a straightforward molecular explanation for the previously reported data (Letourneur *et al.*, 1994; Schröder-Kohne *et al.*, 1998) that shifting *sec27-1* cells to a semirestrictive temperature induces a retrograde transport defect for both a KKTN- and a KTKLL-tagged protein, and defects for the binding to the KKTN motif in vitro. Our data suggest that the *sec27-1* mutation adversely affects interactions of subunits within the coatomer complex to some degree even at permissive temperature.

The WD40 Domains of α- and β'-COP Are Required for Direct Binding to Di-lysine Motifs in the Two-Hybrid System

The yeast two-hybrid system has been successfully used to dissect the specificity of the interaction of the μsubunit of the adaptor complexes AP-1, AP-2, and AP-3 with tyrosine-based trafficking motifs (see e.g., Ohno *et al.*, 1995). In a first attempt to use the two-hybrid system to understand interactions of COP I subunits with di-lysine motifs, we tested the yeast coatomer subunits in the prey vector against several cytoplasmic tails of proteins containing KKTN, or similar motifs, in our standard LexA two-hybrid assay. We observed no specific signals by using this approach (our unpublished data), consistent with the negative results reported by others by using mammalian coatomer subunits in a standard GAL4 two-hybrid system (Zerangue *et al.*, 2001). Interestingly, however, it has been shown previously that fusing an oligomerization domain in frame to short peptide tails containing di-lysine motifs is a technical improvement that can result in specific, positive signals by using such short-peptide two-hybrid constructs (Zerangue *et al.*, 2001). Using this modified two-hybrid system and a strong binding
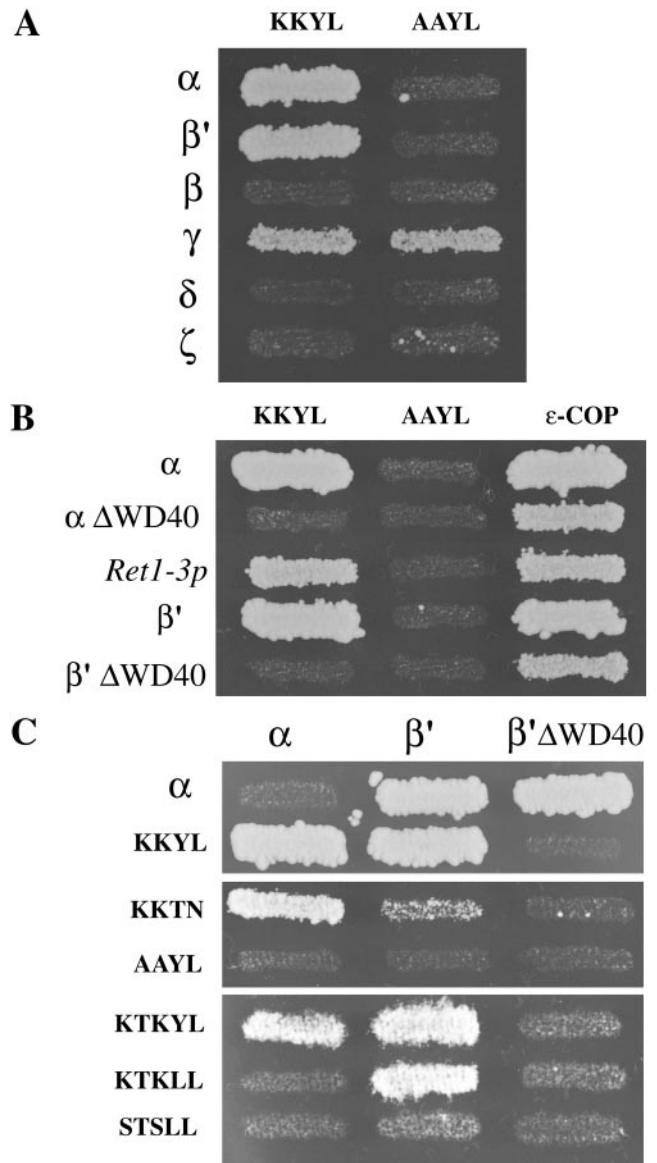

Figure 5. α- and β'-COP directly interact with variants of di-lysine motifs. (A) Coatomer subunits, denoted with Greek symbols, in the bait vector were tested against the KKYL or AAYL motifs in the prey vector. Note that both α- and β'-COP interact with the KKYL motif, in a manner that depends on the presence of the lysines. γ-COP displayed background and thus could not be analyzed. (B) The interaction of α- and β'-COP with the KKYL motif depends on the presence of their WD40 domains. As expected, the interaction of α- and β'-COP with ε-COP is unaffected by truncation of the WD40 domains. "αΔWD40" and "β'ΔWD40" correspond to *ret1Δ1-285* and *sec27Δ1-285* in the text. (C) α-COP, or the different di-lysine motifs, in the bait vector were tested against α-COP, β'-COP, or the β'ΔWD40 mutant in the prey vector. Note that the KKTN motif interacts with α-COP and the KTKLL motif with β'-COP. The presence of the WD40 domain on β'-COP is required for the interaction with KTKLL, but not with α-COP. Results shown are from the growth assay on minimal plates lacking leucine.

variant of the di-lysine motif, KKYL (which does not occur naturally in cells), a specific interaction with mammalian α-COP has been demonstrated by Zerangue *et al.* (2001). The signal in the modified system is thought to be enhanced by an increased effective local concentration of tail sequences

when presented as an oligomeric array (Zerangue *et al.*, 2001).

We used this approach in our LexA two-hybrid system (Eugster *et al.*, 2000) to test interactions of yeast coatomer subunits with the artificially strong di-lysine motif, KKYL, as well as with two naturally occurring di-lysine motifs, KKTN as present on Wbp1p, and KTKLL as present on the cycling Golgi protein Emp47p (Figure 5). We find that the KKYL motif interacts with both α- and β′-COP, whereas no interaction was seen with a mutated motif, AAYL (Figure 5A). No interaction with KKYL was detected with β-, δ-, or ζ-COP, whereas the γ-COP construct showed nonspecific background and thus could not be analyzed (Figure 5A). We next tested whether mutations in α- or β′-COP affect the interaction with the KKYL motif (Figure 5B). A truncated α-COP lacking its WD40 domain (*ret1*Δ1-285) was unable to interact with the KKYL motif, but as expected still interacted with ε-COP used as a control. The *ret1-3* mutation, which resides in the carboxy terminus of α-COP and affects the interaction with ε-COP at restrictive temperature (Eugster *et al.*, 2000), affected neither the interaction with the KKYL motif nor with ε-COP at the permissive temperature (24°C) tested (Figure 5B, *ret1-3*). The two-hybrid results with α-COP are consistent with our previous study in which we demonstrated the importance of the α-COP WD40 domain for interactions with the KKTN motif in vivo and in vitro (Eugster *et al.*, 2000). Similar to α-COP, a truncated β′-COP lacking its WD40 domain (*sec27*Δ1-285) was unable to interact with the KKYL motif, but still interacted with ε-COP used as a control. Therefore, we demonstrate here for the first time the ability of β′-COP to specifically interact with a di-lysine type motif, in a manner that requires the presence of the β′-COP WD40 domain. The fact that the artificial, strong motif KKYL can interact with both α- and β′-COP demonstrates an overlap in the binding activities of α-and β′-COP. However, for naturally occurring and thus weaker signals we find a clear selectivity (Figure 5, B and C). The KKTN motif (as present on Wbp1p) interacts predominantly with α-COP and the KTKLL motif (as present on Emp47p) interacts only with β′-COP, but not α-COP (Figure 5C). Again, as expected these interactions required the presence of the two lysines, because mutating them to alanine or serine abolished them. To test whether the two-hybrid signal for KTKLL motif is, like the KKTN motif, dependent on the local sequence context we also tested the KTKYL motif, which does not occur naturally. This motif could, like the strong KKYL motif, interact with both α-COP and β′-COP. This again highlights some degree of overlap in the binding activities of the WD40 domains. In summary, the WD40 domains of α- and β′-COP can both directly interact with di-lysine motifs but display a preferential interaction with two naturally occurring variants of the di-lysine motif, KKTN and KTKLL, respectively.

The WD40 Domain of β′-COP Is Required for the Correct Localization of the KTKLL-tagged Protein Emp47p

To understand the effect of mutations affecting the WD40 domain of β′-COP on trafficking, we had to extend our analysis to the Golgi protein Emp47p. This type I integral membrane protein maintains its steady-state localization by continuous recycling between the ER and the Golgi complex. This recycling is dependent on its carboxy-terminal KTKLL signal (Schröder *et al.*, 1995; Schröder-Kohne *et al.*, 1998; Sato and Nakano, 2002, 2003). We tested the intracellular fate of Emp47p in α- and β′-COP mutants at a permissive temperature for growth of all mutants tested, to score primary trafficking defects caused by the mutations, rather than sec-

ondary defects that may arise upon temperature shift. We reasoned that if the KTKLL motif is recognized by the β′-COP WD40 domain, as indicated by our two-hybrid data, then mutants affecting this domain may be compromised for Emp47p stability already at a permissive temperature, whereas other coatomer mutants may behave like wild-type cells. Only upon shift to a restrictive temperature would such mutants mislocalize Emp47p. To test this, we first measured the degradation of Emp47p occurring in different mutant cells by using immunoblotting as an assay, as described by Lewis and Pelham (1996). Briefly, wild-type and mutant strains were grown at 24°C. Protein synthesis was inhibited by adding 20 μg/ml cycloheximide to the culture, cultures shifted to 30°C, and then one sample taken immediately and two others after further incubation for 1 and 2 h. Emp47p levels in total cell extracts were analyzed by immunoblot with a rabbit antiserum against Emp47p (Schröder-Kohne *et al.*, 1998) and an antiserum against Sed5p as a control (Figure 6A). In this experiment, a reduction in the level of Emp47p is indicative of its mislocalization to the vacuole where degradation occurs. We tested wild-type cells, the β′-COP mutants *sec27-95*, *sec27*Δ1-285, and *sec27-1*, and the α-COP mutants *ret1-1* and *ret1*Δ1-285 (Figure 6A). Little difference in Emp47p levels between the 0-, 1-, and 2-h time points was found in wild-type and the α-COP mutant cells, consistent with previously published data (Schröder *et al.*, 1995; Schröder-Kohne *et al.*, 1998). Furthermore, the *sec27-1* mutant also behaved similar to wild-type cells. However, in cells of the β′-COP WD40 domain point mutant *sec27-95* and, to a somewhat lesser extent also in the *sec27*Δ1-285 WD40 domain truncation mutant, the level of Emp47p was greatly reduced at the 2-h time point (Figure 6A).

In temperature-shift experiments with yeast, temperature-sensitive mutants secondary defects frequently arise. In previous studies, Emp47p levels had been analyzed only after a preshift to the restrictive temperature for the mutants tested (Schröder *et al.*, 1995; Lewis and Pelham, 1996; Schröder-Kohne *et al.*, 1998). In fact, in cells shifted to restrictive temperature Emp47p has been reported previously to be largely mislocalized to the vacuole in a broad variety of coatomer mutants (*sec27-1* [β′], *sec21-1* [γ], *ret2-1* [δ], and *ret3-1* [ζ]) as well as in other retrograde transport mutants, e.g., *sec20-1* or *ufe1-1* (Schröder-Kohne *et al.*, 1998; Lewis and Pelham, 1996). However, the important exception are the *ret1-1* (α) and similar α-COP mutants in which Emp47p is stable even at restrictive temperature (Schröder-Kohne *et al.*, 1998). Considering these data in the literature together, we hypothesized that the Emp47p mislocalization previously observed in coatomer mutants at high temperature is a consequence of a general coatomer inactivation with the resulting overall shut-down of Golgi-to-ER retrieval. Therefore, such results do not reveal exactly how Emp47p interacts with coatomer.

To obtain quantitative data on Emp47p degradation, pulse-chase experiments were performed with cells grown at 24°C, as described by Schröder-Kohne *et al.* (1998). Briefly, cells were radiolabeled for 20 min, chased for 0, 30, 60 and 120 min and then lysed. Emp47p was recovered with an Emp47p antiserum, resolved by SDS-PAGE, and analyzed using a PhosphorImager. In a first set of experiments, we compared Emp47p stability in *sec27-1*, *sec27-95*, and wild-type cells grown at 24°C (Figure 6B), which was interesting to us because at restrictive temperature *sec27-1* cells display a strong defect for Emp47p (Schröder-Kohne *et al.*, 1998). We find that at 24°C, *sec27-1* cells behaved similar to wild-type, displaying a reduction to 72% of the initial Emp47p level at 120-min chase, compared with 78% in wild-type cells (Fig-
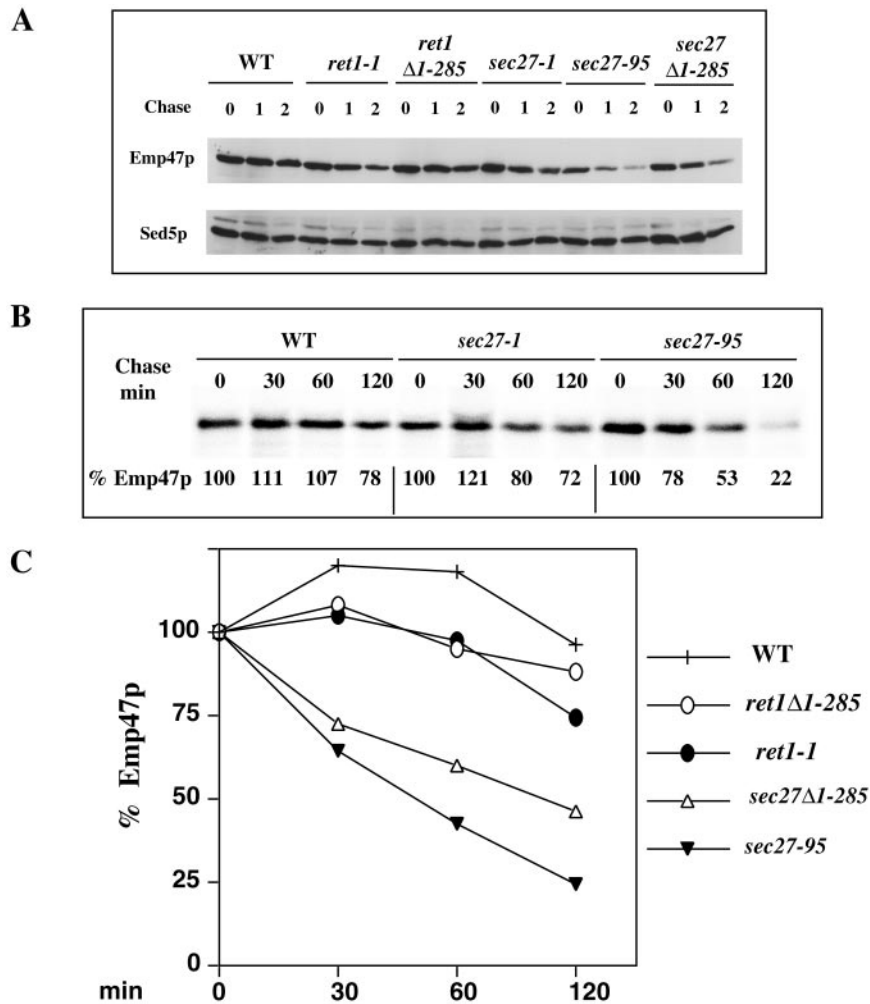
Figure 6. Emp47p is destabilized in β'-COP WD40 domain mutants. (A) Degradation of Emp47p measured by immunoblotting in the strains as indicated; for details, see text. Note a significant reduction of Emp47p levels in cells of the β'-COP WD40 domain mutants *sec27-95* and *sec27Δ1-285*, but not in *sec27-1* cells or *ret1-1* and *ret1Δ1-285* cells. (B) wild-type (wt), *sec27-1* and *sec27-95* cells were pulsed for 20 min at 24°C and chased for the times indicated, and Emp47p levels were quantitated. Note a dramatic reduction of Emp47p levels in *sec27-95* cells but not *sec27-1* cells at 120 min. (C) Turnover of Emp47p in α- and β'-COP WD40 domain mutants. The experiment was performed as in B, by using wild-type cells and the *sec27Δ1-285*, *sec27-95*, *ret1Δ1-285*, *ret1-1* mutants. Curves represent PhosphorImager quantitation of a representative experiment. Note the reduction of Emp47p levels in *sec27Δ1-285* and *sec27-95* cells but not in the α-COP mutants.

ure 6B). A similar result was obtained when testing the γ-COP mutant *sec21-1* at 24°C (our unpublished data). *sec27-95* cells displayed a strong defect even at this permissive temperature, resulting in a dramatic reduction of the Emp47p level to 22% at 120-min chase (Figure 6B).

Next, we compared Emp47p stability in our α- and β'-COP WD40 domain mutants, i.e., *ret1-1*, *ret1Δ1-285*, *sec27-95*, and *sec27Δ1-285* cells, as well as wild-type cells. The data are shown graphically in Figure 6C. Both β'-COP WD40 domain mutants displayed a defect for Emp47p stability, namely, a reduction of Emp47p in *sec27-95* and *sec27Δ1-285* to 24 and 47%, respectively, after 2-h chase. The α-COP WD40 domain mutants behaved similar to wild-type, with values for Emp47p of 95, 90, and 77% for wild-type, *ret1Δ1-285*, and *ret1-1* cells, respectively, after 2-h chase (Figure 6C). On the basis of our data, we conclude that the trafficking of the protein Emp47p, which harbors the variant di-lysine motif KTKLL, is dependent on the WD40 domain of β'-COP rather than α-COP, and therefore β'-COP is required for recruitment of some cargo proteins with a di-lysine type signal at their carboxy terminus.

Mutations in the β'-COP WD40 Domain Do Not Affect KKTN-dependent Trafficking

We next analyzed the ability of the β'-COP WD40 domain mutants *sec27Δ1-285* and *sec27-95* to retrieve a KKTN-tagged protein from the Golgi to the ER in vivo at permissive temperature, 24°C. As a reporter we used a well characterized reporter, Inv-Wbp1p, consisting of invertase fused to the transmembrane and cytoplasmic domains of Wbp1p, a KKTN-tagged protein resident in the ER (Gaynor *et al.*, 1994; Letourneur *et al.*, 1994). In wild-type cells this chimera continuously cycles between the Golgi and the ER, but in coatomer mutants defective for KKTN-dependent retrieval Inv-Wbp1p escapes to the vacuole where it is cleaved, thus enabling a quantitative evaluation of the retrieval defect. It has been previously shown that both the WD40 domain mutants *ret1-1* and *ret1Δ1-285* display a strong KKTN-retrieval defect in vivo, even at permissive temperature (Letourneur *et al.*, 1994; Eugster *et al.*, 2000).

Wild-type cells, and cells of the mutants *sec27Δ1-285*, *sec27-95* cells, *sec27-1*, and *ret1Δ1-285*, all expressing the Inv-Wbp1 fusion, were radiolabeled for 20 min and chased for 0 and 60 min at 24°C. The Inv-Wbp1 fusion was recovered by immunoprecipitation, treated with endoglycosidase H, and resolved by SDS-PAGE (Figure 7A). Sixty percent of the protein was found to be cleaved in *ret1Δ1-285* cells (Figure 7B). In *sec27Δ1-285* cells and *sec27-95* cells only 24% of the protein was processed, very similar to the 21% in wild-type cells (Figure 7B). *sec27-1* cells also behaved like wild-type cells at 24°C (our unpublished data). Even upon shift to 30°C for 3 h, a semipermissive temperature for
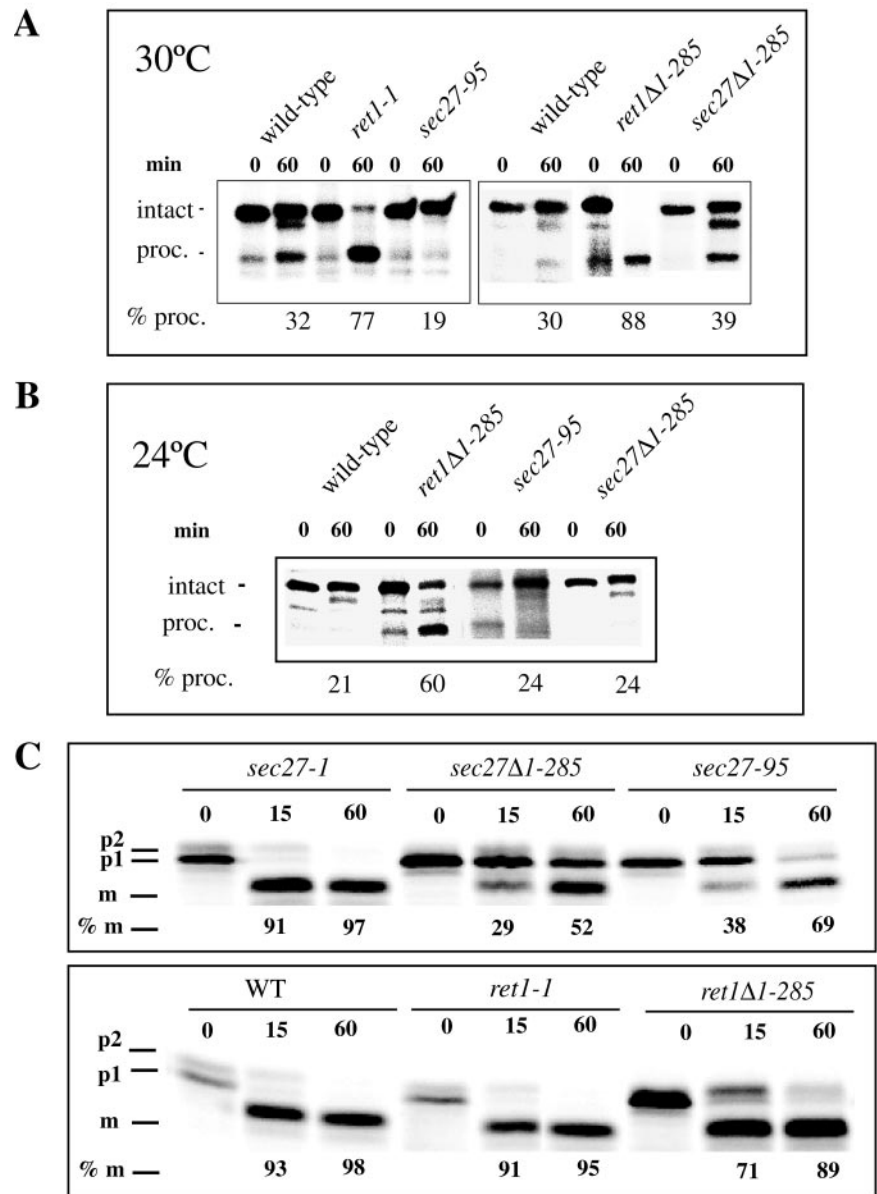
Figure 7. (A) The β′-COP WD40 domain mutants *sec27Δ1-285* and *sec27-95* behave like wild-type for KKTN trafficking in vivo. *sec27Δ1-285* and *sec27-95* cells and *ret1Δ1-285* and wild-type (*CEN::SEC27*) cells as controls, all expressing the Wbp1-Inv.-KKTN fusion construct, were grown at 24°C. Before the experiment cells were preshifted to 30°C for 3 h and then pulse labeled at 30°C, and chased for 0 and 1 h at 30°C. Intact and *PEP4* processed bands migrate at 70 and 56 kDa, respectively. The percentage of processing after 1 h was quantified using a PhosphorImager. (B) Same as in A, except that *ret1Δ1-285*, *sec27Δ1-285*, and control (*CEN::SEC27*) cells were grown at 24°C, pulse labeled for 20 min at 24°C, and chased for 0 and 1 h at 24°C. Note that *ret1Δ1-285* cells show a strong KKTN retrieval defect at both 24 and 30°C, whereas *sec27Δ1-285* cells behave like wild-type. (C) *sec27Δ1-285* and *sec27-95* cells display a kinetic delay in CPY transport at permissive temperature. Analysis of CPY transport in *sec27Δ1-285*, *sec27-95*, *ret1Δ1-285*, *ret1-1*, and wild-type (WT) cells. Cells were radiolabeled for 10 min at 24°C and chased for 0, 15, and 60 min. p1, p2, and mature forms of CPY are indicated, and processing to the mature form is quantified.

sec27Δ1-285, this mutant did not display a strong defect in KKTN-dependent trafficking. At 30°C only 39% of the protein was processed in *sec27Δ1-285* cells, compared with 30% in wild-type cells (Figure 7A). Similar values were obtained for *sec27-95* (Figure 7A, left) and *sec27-1* cells (our unpublished data). Under the same conditions, 77 and 88% of the fusion protein was cleaved in *ret1-1* and *ret1Δ1-285* cells, respectively (Figure 7A).

Our attempts to directly compare the two naturally occurring di-lysine signals, KKTN and KTKLL, with regard to their effect on the maturation of the reporter protein invertase failed due to the general weakness of the KTKLL signal noticed in earlier studies (Schröder-Kohne *et al.*, 1998). Specifically, we generated KTKLL-/STSLL-tagged invertase reporter proteins in an attempt to compare them with the KKTN invertase reporter (Gaynor *et al.*, 1994) used here. However, even in wild-type cells the KTKLL-tagged invertase reporter was rapidly transported to the vacuole and degraded, indistinguishable from un-

tagged invertase reporter (our unpublished data), and therefore failed to provide an assay for KTKLL-mediated recycling.

To estimate the degree to which the β′-COP WD40 domain mutants can support normal anterograde transport of proteins in the secretory pathway at permissive temperature, 24°C, we analyzed the transport of the vacuolar protease CPY from the ER to the vacuole. We find that the β′-COP WD40 domain mutants *sec27-95* and *sec27Δ1-285* display a modest kinetic delay in the maturation of CPY, whereas *ret1-1*, *ret1Δ1-285*, and the *sec27-1* mutants behaved like wild-type cells (Figure 7C). This difference between the two β′-COP mutants in their ability to perform forward transport (i.e., with *sec27Δ1-285* cells affected more strongly with regard to CPY transport than *sec27-95* cells) explains in a straightforward manner why Emp47p is less strongly mislocalized in *sec27Δ1-285* cells compared with *sec27-95* cells, because forward transport of Emp47p is a prerequisite for its degradation in the vacuole.

Table 2. Summary of phenotypes of β'- and α-COP mutants

Alleles	Changes	Defects in vivo/in vitro	References
sec27-1 (β'-COP)	G688D	ts; no KKTN transport defect and no defect for Emp47p (KTKLL) stability at permissive temperature; after ts-shift α-COP instability and both KKTN and KTKLL transport defects	This study; Duden *et al.*, 1994; Letourneur *et al.*, 1994; Schröder-Kohne *et al.*, 1998
sec27-95 (β'-COP)	S114Y	ts; strong defect for Emp47p (KTKLL) stability; no KKTN transport defect	This study; Prinz *et al.*, 2000
sec27Δ1-285 (β'-COP)	residues 1-285 deleted	ts; strong defect for Emp47p (KTKLL) stability; no KKTN transport defect; defective for binding KKTN and KTKLL motifs in vitro	This study
ret1-1 (α-COP)	G227D	weak ts; no defect for Emp47p (KTKLL) stability, strong KKTN transport defect; defective for binding to KKTN, but able to bind KTKLL motifs in vitro	Letourneur *et al.*, 1994; Schröder-Kohne *et al.*, 1998
ret1Δ1-285 (α-COP)	residues 1-285 deleted	ts; no defect for Emp47p (KTKLL) stability, strong KKTN transport defect; defective for binding KKTN and KTKLL motifs in vitro	This study; Eugster *et al.*, 2000

ts, temperature sensitive.

In summary, in contrast to *ret1Δ1-285* and *ret1-1* cells, neither of the three β'-COP mutants display a defect in the retrieval of a KKTN-tagged protein either at permissive temperature or upon shift to the semipermissive temperature, 30°C. The trafficking phenotypes of the α- and β'-COP mutants with regard to proteins harboring KKTN- or KTKLL-signals are summarized in Table 2.

Binding of Coatomer from α- and β'-COP Mutants to Di-lysine Motifs In Vitro

To directly assess the ability of coatomer from β'-COP mutant cells to interact with di-lysine motifs, in vitro binding experiments were performed using GST-fusion proteins harboring the cytoplasmic tail of Wbp1p, which terminates in a "classical" di-lysine motif, KKTN, and the cytosolic tail of Emp47p, which terminates in a variant di-lysine motif, KTKLL. GST fusions in which the lysines were mutated to serines were used as controls. These experiments used whole cell extracts from cells grown at 24°C, and the high salt/0.5% Triton X-100 binding conditions described by Cosson and Letourneur (1994). We analyzed extracts from the mutants *sec27-95*, *sec27Δ1-285*, ret1-1, and *ret1Δ1-285*, and from wild-type cells. We find that wild-type coatomer bound to both KKTN- and KTKLL-GST and *ret1-1* coatomer only to KTKLL-GST, as reported previously (Schröder-Kohne *et al.*, 1998). Coatomer from the truncation mutants *sec27Δ1-285* and *ret1Δ1-285* was unable to bind to either motif. Coatomer from *sec27-95* cells showed only weak binding to KTKLL-GST and no binding to the KKTN motif (Figure 8). We speculate that coatomer lacking one of its WD40 domains or from *sec27-95* cells failed to withstand the rather harsh incubation/washing conditions required to reveal binding of wild-type or *ret1-1* coatomer to the di-lysine tagged GST fusions in a significant manner (Schröder-Kohne *et al.*, 1998). On the other hand, in vivo the correct targeting of a KKTN-dependent reporter and of the endogenous KTKLL-tagged protein Emp47p clearly depends on one of two WD40 domains of coatomer in a mutually independent manner, as shown above.

DISCUSSION

We wish to better understand the interaction between vesicle cargo proteins and the COP I/coatomer coat. The WD40 domain of α-COP has been previously shown to be required for the binding and trafficking of proteins tagged with the classical di-lysine retrieval motif KKTN (Schröder-Kohne *et al.*, 1998; Eugster *et al.*, 2000). Here, we studied the role of the WD40 domain of β'-COP in the recognition of the variant di-lysine motif, KTKLL. To this end we analyzed two known point mutations (*sec27-1* and *sec27-95*) and a truncated version (*sec27Δ1-285*) of β'-COP with regard to their effects on the binding and trafficking of proteins tagged with these two
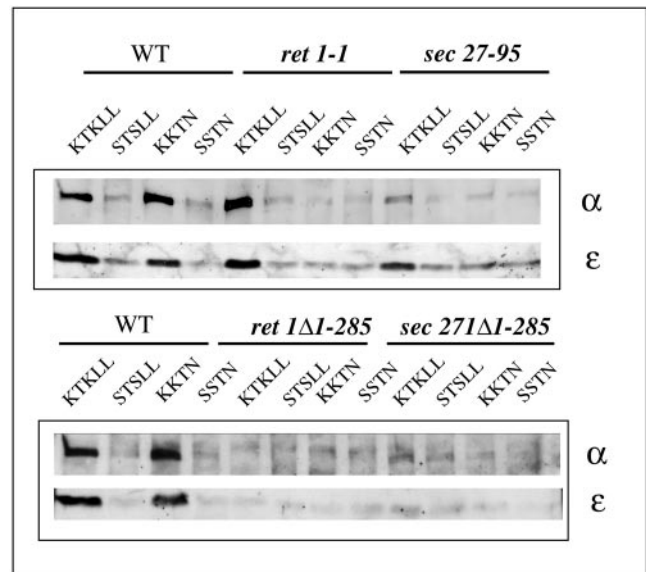

Figure 8. Coatomer from WD40 domain truncation mutants is unable to bind to either the KKTN- or KTKLL motifs in vitro. Whole cell lysate of wild-type cells (WT), or mutants (*ret1Δ1-285* cells, *ret1-1* cells, *sec27-95*, *sec27-1*, *sec27Δ1-285*) was incubated for 2 h at 4°C with immobilized GST fusion proteins harboring the KKTN- or KTKLL motifs, or mutated motifs in which the lysines were changed to serine, SSTN or STSLL. Bound proteins eluted from the beads were analyzed by immunoblot. Note the expected binding pattern of coatomer from wild-type and *ret1-1* mutant cells, and the absence of binding for coatomer from the α- and β'-COP WD40 domain truncation mutants. Coatomer from *sec27-95* cells bound only weakly to KTKLL and not at all to the KKTN motif.

di-lysine motifs. Our data demonstrate that the WD40 domain of β′-COP can directly bind the variant di-lysine signal KTKLL and thus is involved in cargo selection and that a carboxy-terminal domain on β′-COP contributes to the structural integrity of the coatomer complex.

A Carboxy-Terminal Region of β′-COP Is Involved in Maintaining Coatomer Integrity

Sequencing of the β′-COP mutant *sec27-1* revealed a G688D mutation in the Clathrin Heavy Chain Repeat of β′-COP (Table 2 and Figure 1). The clathrin heavy chain protein consists of three distinct domains. A terminal domain is responsible for interaction with adaptor complexes, whereas the proximal and distal domains provide the clathrin leg that forms the characteristic outer layer of clathrin-coated vesicles (Kirchhausen, 2000). The clathrin leg consists of seven copies of the Clathrin Heavy Chain Repeat (Ybe *et al.*, 1999). Single copies of this repeat have been found in a number of proteins involved in membrane traffic, including α-COP (Conibear and Stevens, 1998) and β′-COP (Eugster *et al.*, 2000) (Figure 1). Biochemical characterization of *sec27-1* revealed a reduced stability of the α-COP subunit, to some degree even at permissive temperature, and a loss of the α-, β′-, ε-COP subcomplex from the coatomer complex. This explains the rather general nature of coatomer-related trafficking phenotypes found in previous studies by using this mutant, which were performed upon shift to semipermissive or restrictive temperatures (Letourneur *et al.*, 1994; Schröder-Kohne *et al.*, 1998). Further analysis of the *sec27-1* mutant may help to understand the role of the clathrin heavy chain repeat in a nonclathrin complex in molecular detail.

β′-COP WD40 Domain Mutants Mislocalize the KTKLL-tagged Protein Emp47p

α- and β′-COP share a close sequence similarity extending from residues 1–780 (Eugster *et al.*, 2000). Both proteins harbor highly conserved WD40 domains in their amino-terminal regions, and unique regions with no sequence relationship are found at their carboxy termini (Figure 1). To understand the role of the WD40 domain of β′-COP in COP I-mediated trafficking, we had to extend our analysis to the Golgi protein Emp47p. This type I integral membrane protein maintains its steady-state localization by continuous recycling between the endoplasmic reticulum and the Golgi complex (Schröder *et al.*, 1995; Schröder-Kohne *et al.*, 1998; Sato and Nakano, 2002, 2003). This recycling is dependent on its carboxy-terminal variant di-lysine signal, KTKLL (Schröder *et al.*, 1995; Schröder-Kohne *et al.*, 1998). We show here that deletion of the β′-COP WD40 domain or its inactivation by the point mutation of *sec27-95* cells results in loss of Emp47p from the Golgi complex. The recycling of a KKTN-tagged reporter protein, on the other hand, is unaffected in these cells (Table 2). Our findings thus provide an explanation for earlier observations that the KTKLL-tagged protein Emp47 is not mislocalized in α-COP WD40 domain mutants (Schröder *et al.*, 1995; Schröder-Kohne *et al.*, 1998). Apparently, this variant di-lysine motif is dependent on the WD40 domain on β′-COP rather than on α-COP, and therefore β′-COP is required for recruitment of some cargo proteins with a di-lysine–related signal at their carboxy terminus into COP I vesicles.

WD40 Domains as Recognition Modules for Variants of the Di-lysine Motif

The WD40 repeat was originally identified in the β-subunit of heterotrimeric G proteins (Gβ) (Fong *et al.*, 1986).

Later, it was found in >160 functionally diverse proteins by sequence analysis, including the COP II coat proteins Sec13p and Sec31p (Shaywitz *et al.*, 1997) and the COP I proteins α- and β′-COP (Letourneur *et al.*, 1994; Duden *et al.*, 1994; Gerich *et al.*, 1995). The crystal structure of Gβ revealed a propeller-like fold for the WD40 domain (Wall *et al.*, 1995). A WD40 repeat consists of four β-strands arranged in an antiparallel β-sheet, providing one of the blades to the overall propeller-like structure. This β-propeller derives rigidity from hydrophobic interactions between the β-sheets, whereas solvent-exposed residues between β strands mediate interactions with other proteins (Smith *et al.*, 1999).

Using a modified version of the two-hybrid system, we show that the WD40 domains of α- and β′-COP are capable of direct interactions with two naturally occurring variants of the di-lysine motif, namely, KKTN and KTKLL (Figure 5). Interestingly, this direct and specific interaction of a short peptide motif is dependent on the presence of a tetramerization domain in the construct presenting the di-lysine signal, as described earlier by Zerangue *et al.* (2001). We find that the classic di-lysine signal KKTN derived from the oligosaccharyl transferase subunit Wbp1p binds preferentially to the WD40 domain of α-COP. The variant di-lysine signal KTKLL found on the Golgi protein Emp47p binds exclusively to the WD40 domain of β′-COP. The strong artificial di-lysine signal KKYL, however, which has been identified in a screen for functional di-lysine signals (Zerangue *et al.*, 2001), is able to interact with both WD40 domains with high affinity. Another artificial di-lysine signal, KTKYL, can also interact with both WD40 domains, albeit with lower affinity. These data highlight some degree of overlap in the binding activities of the two WD40 domains. However, our two-hybrid data also clearly show that the two WD40 domains of α- and β′-COP are able to discriminate between two naturally occurring di-lysine signals. The trafficking phenotypes observed in a panel of α- and β′-COP WD40 domain mutants are consistent with this notion. Another interaction site in coatomer for di-lysine type motifs has been indicated in mammalian γ-COP by using a photo-cross-linking approach (Harter *et al.*, 1996; Harter and Wieland, 1998) and by in vitro binding data (Wu *et al.*, 2000). However, this binding site must be structurally different from the binding sites on α- and β′-COP reported here, because γ-COP does not harbor WD40 repeats.

An increasing number of WD40 domain-containing proteins have been found to interact with short sequences or small structural motifs with high specificity (Beisel *et al.*, 1999; Holm *et al.*, 2001). For example, a regulatory protein from *Arabidopsis thaliana* with a seven bladed β-propeller formed by WD40 repeats has been reported to bind a variety of ligands presenting the binding motif V-P-E/D-Φ-G; (with Φ being a bulky hydrophobic residue) in conjunction with four to five negatively charged residues upstream of the motif (Holm *et al.*, 2001). X-ray crystallography on the lectin Tachylectin-2 in a complex with its ligand revealed one N-acetyl carbohydrate-binding site per blade, i.e., five binding sites in a five bladed β-propeller (Beisel *et al.*, 1999). Structural studies will have to show whether there are multiple binding sites for di-lysine motifs on each of the WD40 domains in coatomer.

In summary, we have demonstrated that the α-COP WD40 domain directly interacts with the classical KKTN-motif, whereas the β′-COP WD40 domain directly interacts with a variant di-lysine motif, KTKLL. Our data

suggest that the WD40 domains of α- and β'-COP bind distinct but overlapping sets of di-lysine signals and that both domains contribute to recruitment of cargo proteins with di-lysine motifs into COP I vesicles. Similarly, a variety of ER export signals are recognized by subunits of the COP II coat in a manner that also involves multiple, distinct cargo recognition sites (for review, see Barlowe, 2003). It will be interesting to analyze further COP I cargo proteins with regard to their direct interactions with α- or β'-COP: Rer1p, a recycling receptor for the transmembrane protein Sec12p that is required for COP II vesicle budding (Sato *et al.*, 2001), Golgi-resident type II integral membrane proteins such as Och1p (Harris and Waters, 1996), or the HDEL/KDEL-receptor Erd2p (Townsley *et al.*, 1993). Our WD40 domain mutants of α- and β'-COP should enable us to study the trafficking of COP I cargo proteins with regard to their binding site on coatomer. Analysis of the exact ligand specificity of the WD40 domains will require the use of recombinant, soluble WD40 domains for in vitro binding assays. This approach may allow characterization of the interaction of these domains with di-lysine-type motifs in structural detail.

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