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Novel cargo-binding site in the β and δ subunits of coatomer

Kai Michelsen,[1] Volker Schmid,[1] Jutta Metz,[1] Katja Heusser,[1] Urban Liebel,[2] Torsten Schwede,[3] Anne Spang,[4] and Blanche Schwappach[1]

[1]Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany
[2]European Molecular Biology Laboratory (EMBL), Cell Biology Cell Biophysics Unit, D-69117 Heidelberg, Germany
[3]Swiss Institute of Bioinformatics, Biozentrum, University of Basel, CH-4056 Basel, Switzerland
[4]Growth & Development, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Arginine (R)-based ER localization signals are sorting motifs that confer transient ER localization to unassembled subunits of multimeric membrane proteins. The COPI vesicle coat binds R-based signals but the molecular details remain unknown. Here, we use reporter membrane proteins based on the proteolipid Pmp2 fused to GFP and allele swapping of COPI subunits to map the recognition site for R-based signals. We show that two highly conserved stretches—in the β- and δ-COPI subunits—are required to maintain Pmp2GFP reporters exposing R-based signals in the ER. Combining a deletion of 21 residues in δ-COP together with the mutation of three residues in β-COP gave rise to a COPI coat that had lost its ability to recognize R-based signals, whilst the recognition of C-terminal di-lysine signals remained unimpaired. A homology model of the COPI trunk domain illustrates the recognition of R-based signals by COPI.

Introduction

The sorting of membrane proteins relies on different peptide-sorting motifs that are recognized by vesicle coats (like COPI, COPII, or clathrin) and their adaptor complexes. These interactions can capture cargo at various donor membranes (e.g., the ER, different Golgi compartments, or the plasma membrane), and lead to their inclusion into transport vesicles. Only for a few peptide-sorting motifs (e.g., motifs of the YXXΦ class that mediate the endocytosis of cargo proteins) is the interaction with the relevant clathrin adaptor complex understood in structural detail (Owen et al., 2004), whereas for many even the subunit harbouring the binding site remains unknown. ER localization signals comprising a C-terminal di-lysine motif (K(X)KXX) are thought to bind to the WD40 domains of the α- and β′-COPI subunits (Eugster et al., 2004). In contrast, it is completely unknown how other classes of ER localization motifs (e.g., R-based signals) are recognized. These signals occur in a growing list of cell surface receptors and ion channels (Michelsen et al., 2005). Many of these multimeric membrane proteins exert critical functions and therefore their assembly is tightly controlled to allow only multimers of a specific composition to reach the cell surface. Incompletely or incorrectly assembled complexes are retrieved back to the ER. In monomeric proteins R-based signals act as ER localization signals, but in assembled multimeric proteins they can be hidden in the complex or rendered inactive by the recruitment of 14-3-3 proteins (Zerangue et al., 1999, 2001; O'Kelly et al., 2002; Yuan et al., 2003; Brock et al., 2005; Heusser et al., 2006; Mrowiec and Schwappach, 2006). Previous work clearly implicates the COPI coat as an important player in the recognition of R-based signals: COPI was shown to bind to different types of R-based signals present in the two-pore domain potassium channel KCNK3 or to one subunit of the heterodimeric G protein–coupled receptor $GABA_B$ (O'Kelly et al., 2002; Brock et al., 2005). Yuan et al. (2003) showed specific (R-based signal-dependent) binding of purified mammalian coatomer to the tail of the inwardly rectifying potassium channel Kir6.2, the pore-forming subunit of ATP-sensitive potassium channels. Because coatomer forms a tight heptameric complex these experiments did not reveal the subunit containing the binding site for R-based signals. Having recently shown that R-based sorting motifs are functional in yeast (Michelsen et al., 2006), we embarked on a reverse genetic screen to characterize which genes are required for the function of R-based signals as ER localization signals. In addition, we dissected the role of COPI in the recognition of R-based signals. Our results

Correspondence to Blanche Schwappach: b.schwappach@zmbh.uni-heidelberg.de
U. Liebel's present address is Institute of Toxicology and Genetics, Forschungszentrum Karlsruhe, D-76021 Karlsruhe, Germany.

Abbreviations used in this paper: AD, activation domain; BD, binding domain.

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demonstrate that combinatorial interaction with different binding sites may play an important role in the dynamic interplay between sorting machinery and membrane cargo proteins.

Results and discussion

COPI-mediated retrieval is the main mechanism responsible for the activity of R-based signals as ER localization signals

We screened a deletion mutant library covering all nonessential *Saccharomyces cerevisiae* genes (Winzeler et al., 1999) and a yeast strain collection harboring essential genes under the control of a regulatable promoter (Mnaimneh et al., 2004). We fused the last 36 residues of the mammalian inwardly rectifying potassium channel Kir6.2 to Pmp2, a single-spanning (type I) yeast membrane protein, to obtain Pmp2GFP-LRKR. As shown previously (Michelsen et al., 2006), the reporter localized to the ER due to the exposure of the well-characterized R-based signal present in the Kir6.2 tail (Zerangue et al., 1999). We transformed a plasmid encoding the Pmp2GFP-LRKR reporter into both strain collections and analyzed the resulting transformants by light microscopy (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200704142/DC1). Inactivation of only 13 genes was found to affect the steady-state ER localization of the reporter (Table I). To further characterize the hits, a plasmid encoding Pmp2GFP-KKTN (Michelsen et al., 2006) was introduced into the strains identified in the screen (Fig. S1 A; and unpublished data). In wild-type cells, Pmp2GFP-KKTN localizes to the ER due to the exposure of the C-terminal di-lysine (KKXX) signal (Michelsen et al., 2006). R-based sorting motifs and KKXX signals fall into distinct classes because the details of how they are recognized are not identical (Shikano and Li, 2003; Michelsen et al., 2005). The ER localization of Pmp2GFP-LRKR and Pmp2GFP-KKTN was compromised in all candidate strains (Table I, Fig. S1 A; and unpublished data). Strikingly, several genes identified in the screen coded for COPI subunits or subunits of the Dsl1/Dsl3 tethering complex that is thought to be part of the ER target site for COPI-coated vesicles (Andag et al., 2001; Reilly et al., 2001). All but one coatomer subunit and the members of the Dsl1/Dsl3 tethering complex are essential. Hence, it is not surprising that the shut-off of these genes affects both types of cargo.

Because COPI functions as a heptameric complex, the genes encoding the α-, ϵ-, and ζ-COP subunit should have been identified in the screen. We investigated the localization of Pmp2GFP-LRKR and Pmp2GFP-KKTN in the corresponding deletion or shut-off strains (Fig. S1, B and C). The result shows that all COPI subunits but ϵ-COP (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200704142/DC1) are required for efficient ER localization of Pmp2GFP-LRKR. This result is consistent with the fact that ϵ-COP is the only dispensable COPI subunit (Duden et al., 1998). Presumably the genes encoding α- and ζ-COP were missed in the screen because of incomplete down-regulation. Similarly, the gene encoding Dsl1 that associates with Dsl3 to form a tethering complex thought to be involved in the fusion of retrograde vesicles with the ER was not detected in the screen, although the gene encoding Dsl3 was (Table I, Fig. S1 A). Consistent with the proposed role for a complex containing Dsl1 and Dsl3 in COPI-mediated retrograde transport, we found that down-regulation of the *DSL1* gene resulted in the mislocalization of Pmp2GFP-LRKR and Pmp2GFP-KKTN (Fig. S1 B). The accumulation of coatomer subunits in the screen strongly suggests an intimate relationship between the sorting of membrane proteins exposing an R-based signal and coatomer. Therefore, we decided to concentrate on the role of COPI in the recognition of R-based signals.

Short conserved stretches in the δ- and β-COP subunits interact with R-based signals

The two WD40 domains of α- and β′-COP bind distinct but overlapping sets of di-lysine signals, and hence, both contribute to the recycling of proteins with di-lysine signals (Eugster et al., 2004). To assess whether coatomer discriminates between Pmp2GFP-LRKR and Pmp2GFP-KKTN, we tested the localization of both reporters in a mutant containing an allele with a mutation in the region of α-COP that encodes the WD40 domain (Fig. S2 B). Confirming previous results (Schroder et al., 1995; Eugster et al., 2004), we observed mislocalization of Pmp2GFP-KKTN, whereas Pmp2GFP-LRKR localized robustly to the ER.

Table I. **Genes identified by the reverse genetic screen in *S. cerevisiae***

Mutant	Gene	Phenotype	Description
tetO7::DSL3	*YLR440C*	*sec*-like	*DSL* complex involved in retrograde transport; related to COPI
tetO7::SEC15	*YGL233W*	*sec*-like	exocyst complex component
tetO7::TAF12	*YDR145W*	*sec*-like	TATA binding protein-associated factor
tetO7::SEC26	*YDR238C*	*sec*-like	COPI subunit beta
tetO7::SEC27	*YGL137W*	*sec*-like	COPI subunit beta′
tetO7::SEC65	*YML105C*	*sec*-like	Subunit of the signal recognition particle (SRP)
tetO7::SEC21	*YNL287W*	*sec*-like	COPI subunit gamma
tetO7::ERG26	*YGL001C*	*sec*-like	Ergosterol biosynthesis
tetO7::STH1	*YIL126W*	*sec*-like	helicase related protein; *SNF2* homologue
tetO7::RET2	*YFR051C*	*sec*-like	COPI subunit delta
tetO7::RSC6	*YCR052W*	*sec*-like	Chromatin remodelling; homologue of SWI/SNF subunit *SWP73*
ser33::kanMX4	*YIL074C*	Perinuclear accumulation	serine and glycine biosynthesis
snf2::kanMX4	*YOR290C*	Perinuclear accumulation	SWI/SNF chromatin remodelling complex
pac10::kanMX4	*YGR078C*	Perinuclear accumulation	Biogenesis of microtubuli

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These experiments support the notion that the binding site for R-based signals present in the COPI complex is distinct from the WD40 domain of α-COP that recognizes the KKTN motif (Eugster et al., 2004).

The yeast two-hybrid system has been successfully used to detect the interaction between adaptor subunits and tyrosine-based cargo sorting motifs and between K(X)KXX motifs and COPI subunits (Ohno et al., 1995, 1998; Zerangue et al., 2001; Eugster et al., 2004). We used fusions to the Gal4 DNA-activation domain (AD) presenting a C-terminal tail with a very strong R-based signal (KLRRRRI) or one of low efficacy (NVRNRRK). Both synthetic signals were originally identified in a combinatorial screen of signal variants in mammalian cells (Zerangue et al., 2001). Their differential strength was revealed by quantitative trans-Golgi network processing and cell surface expression assays. Fusions of COPI subunits to the Gal4 DNA-binding domain (BD) (Faulstich et al., 1996) were used to assay the putative interaction between the R-based ER sorting motif and individual COPI subunits (Fig. 1 A). Two plasmids encoding the Gal4-BD fusions to β-COP or to δ-COP gave rise to specific activation of the *HIS3* reporter gene (Fig. 1 A, "interaction test") when tested against the strong variant of the R-based sorting motif.

We used the yeast two-hybrid assay to narrow down the sequence stretch on each COPI subunit that was required to observe a specific interaction with the strong R-based signal. A series of C-terminal deletion mutants of the Gal4-BD fusion to the δ-COP subunit was tested against the strong and the weaker R-based sorting motif (Fig. 1 B). Two constructs ending with residue 399 or 388 marked the transition from a δ-COP protein that was able to recognize R-based signals to one that was not (Fig. 1 B). We aligned the surrounding region of bovine, yeast, human, and fly δ-COP orthologues (Fig. 1 C) and found the region to be highly homologous, consistent with the evolutionarily conserved recognition of R-based sorting motifs (Michelsen et al., 2006). We deleted the conserved region (aa 388 to 413) from the full-length δ-COP Gal4-BD fusion construct and tested whether it had lost its ability to interact with the R-based sorting motif, which was indeed the case (Fig. 1 B). The internal deletion mutant of δ-COP had retained part of its function because δ-COP (Δ388-413) was still capable of binding β-COP in a two-hybrid assay (unpublished data).

β- and δ-COP are known to interact tightly in the trunk domain of the adaptor-like subcomplex (Faulstich et al., 1996; Eugster et al., 2000). To determine the region on the β-COP subunit required for the interaction with the R-based signal, we tested a series of constructs encoding N-terminally truncated forms of β-COP. Removing the N-terminal 210 aa had no effect on the specific interaction, whereas the signal was lost upon deletion of further 156 aa (Fig. 1 D). The responsible region was narrowed down to aa 318–338. A β-COP subunit lacking these residues was incapable of interacting with the strong R-based signal as assayed by the yeast two-hybrid system. As observed for δ-COP, alignment of the corresponding stretch with β-COP orthologues from yeast, human, mouse, and fly revealed high

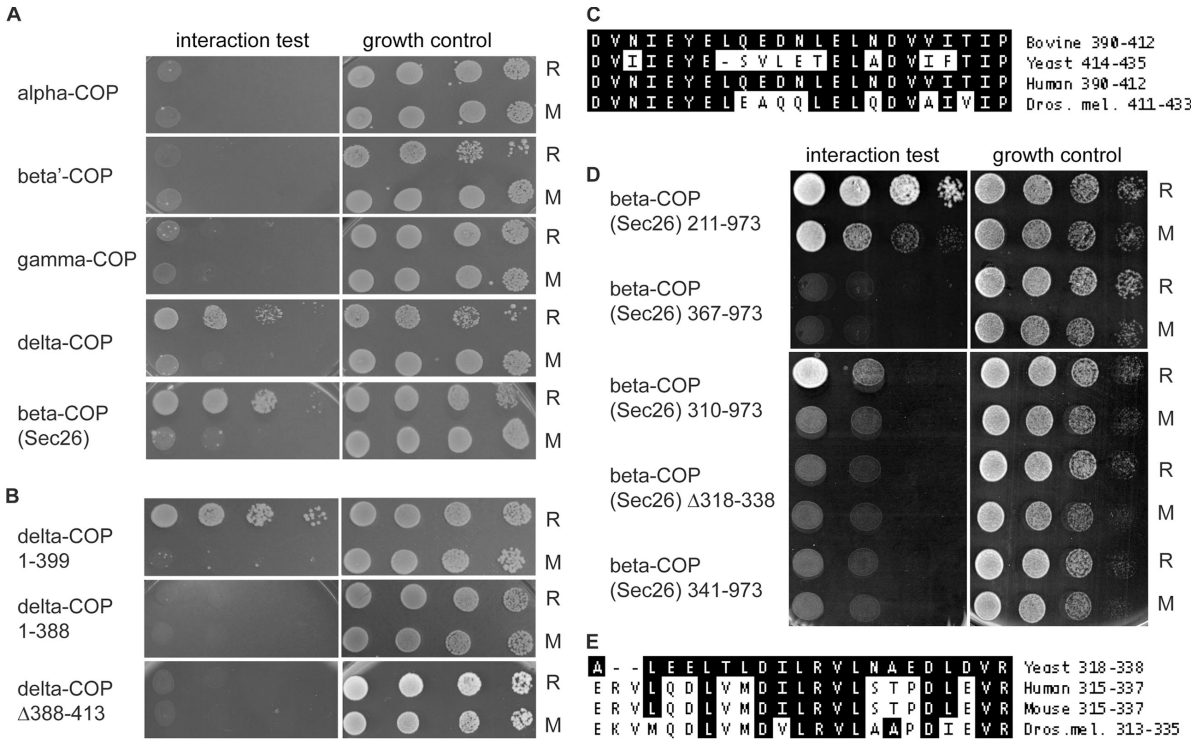

Figure 1. **Identification of regions required for the interaction with R-based signals in the δ- and β-COP subunit.** (A) Yeast two-hybrid analysis of Gal4 BD fusions of five COPI subunits and a strong (R; KLRRRRI) or weak (M; NVRNRRK) R-based signal fused to the Gal4 AD performed at low stringency. Except for β-COP (yeast *SEC26*) all open reading frames encoding COPI subunits were bovine (Faulstich et al., 1996). (B) C-terminal truncation and internal deletion analysis of bovine δ-COP using the yeast two-hybrid assay. (C) Alignment of the critical region as mapped in bovine δ-COP with the corresponding regions of yeast, human, and fly δ-COP proteins. (D) N-terminal truncation and internal deletion analysis of β-COP using the yeast two-hybrid assay. (E) Alignment of the critical region as mapped in yeast β-COP with the corresponding regions of human, mouse, and fly β-COP proteins.

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conservation of the putative binding site (Fig. 1 E). Our yeast two-hybrid analysis implicated the β- and δ-subunit of COPI as candidates harboring the binding site for R-based signals. This result could be explained by different hypotheses: (1) Both COPI subunits contain independent binding sites for the R-based signal; or (2) Both COPI subunits contribute to a common binding site and the affinity of the partial binding pocket is sufficient for a positive signal in the assay.

Two regions in β- and δ-COP are collectively required to recognize R-based signals in vivo

To distinguish between these possibilities, we created a strain that expresses a variant of the COPI coat specifically incapable of recognizing R-based sorting motifs. We exchanged the gene encoding β-COP against *SEC26*^{334}DLD/NAN, (β-COP*) in the critical region and δ-COP against *RET2*Δ414-435 (δ-COP*). Strains expressing β-COP*, δ-COP*, or both were viable, although the double-mutant strain grew slowly and was temperature sensitive. These strains enabled us to test the localization of different reporters in the absence of the putative binding site(s).

We then assessed the functional consequence of removing the two putative binding sites for R-based signals individually and in combination by determining the subcellular localization of Pmp2YFP reporters presenting different variants of the R-based signal (Fig. 2). In mammalian cells, the two tails containing the sequences KLRRRRI or NVRNRRK were shown to function respectively as a very strong and a weak R-based ER localization signal by two independent quantitative assays (see Fig. 4 in Zerangue et al., 2001). Although these results show that the CD4-GFP reporter exposing the weak signal was able to leave the ER, most of the protein localized to the ER in the steady state as assessed by the GFP staining pattern in HeLa cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200704142/DC1). Consistent with the strict conservation of the relative efficacy of R-based signals in yeast (Michelsen et al., 2006), Pmp2YFP-NVRNRRK was also localized in the ER at steady state in wild-type yeast cells (Fig. 2 A). In the strains expressing either β-COP* or δ-COP*, Pmp2YFP-NVRNRRK (exposing a weak R-based signal) was no longer retained in the ER and efficiently reached the vacuole (Fig. 2 A, arrows), whereas Pmp2YFP-KLRRRRI (exposing a strong R-based signal) was still maintained in the ER

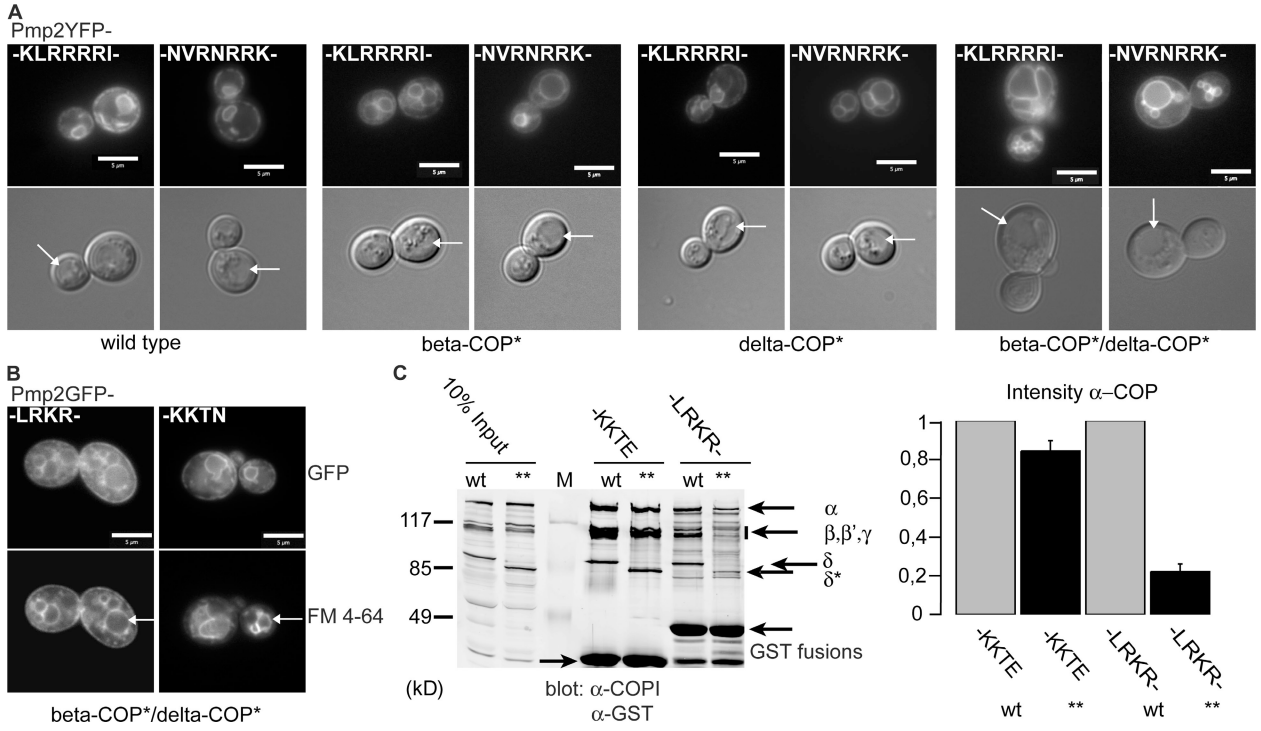

Figure 2. **Specific loss of R-based signal-dependent sorting in cells with mutant coatomer.** (A) Localization of the Pmp2YFP reporter protein presenting a strong (KLRRRRI) or a weak (NVRNRRK) R-based signal in wild-type and cells expressing mutant β-COP* (Sec26 ^{334}DLD/NAN), δ-COP* (Ret2Δ414-435), or both. Cells were grown at 25°C. Top panels: YFP fluorescence, bottom panels: DIC images. Bar, 5 μm. White arrows mark the vacuole. (B) Yeast mutants coexpressing expressing β-COP* and δ-COP* (Sec26 ^{334}DLD/NAN and Ret2Δ414-435; see methods for details of strain construction) and either Pmp2GFP-LRKR or Pmp2GFP-KKTN were stained with FM4-64 as described by Vida and Emr (1995). Top panels: GFP fluorescence, bottom panels: FM4-64 images. Bar, 5 μm. White arrow marks the vacuole. (C) Binding of wild-type (wt) and double-mutant (**) coatomer from yeast cytosol to GST fusion proteins of the cytosolic tail of Mst27 (Sandmann et al., 2003) presenting the indicated sorting signal (KKTE or LRKR). The bound fraction was eluted from the beads and analyzed by SDS PAGE and Western blotting. Blots were probed using an antiserum recognizing all COPI subunits (bands from top to bottom: α-COP/Ret1, an unresolved triplet of β-/Sec26, β'/Sec27, and γ-COP/Sec21, and δ-COP/Ret2; compare Sandmann et al. (2003) for a characterization of the antibodies) and subsequently with an antiserum recognizing the GST-fusion protein immobilized on the glutathione agarose (labeled GST fusions). The signal of the fluorescent secondary antibody was quantified (one representative blot, left). The bar diagram (right) shows the quantification of the α-COP band for seven independent experiments. The intensity of the α-COP signal was normalized to the intensity of the corresponding anti-GST signal to correct for differences in the amount of protein-loaded beads. For each cytosolic tail, the α-COP signal obtained in the pull-down was expressed as a fraction of the signal obtained from the wild-type coat.

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(completely in the strain expressing δ-COP* and substantially in the strain expressing β-COP*; Fig. 2 A). Vacuolar localization is an indication of ER exit and loss of R-based signal recognition because Pmp2 reporters with inactive R-based signals efficiently reach the vacuole (Michelsen et al., 2006). In the strain coexpressing the two altered COPI-subunits, both R-based signal-exposing reporters strongly accumulated in the vacuole as demonstrated by differential interference contrast and colocalization with the fluorescent dye FM4-64 (Fig. 2, A and B). FM4-64 is taken up from the plasma membrane and reaches the vacuole via the endocytic route (Vida and Emr, 1995). In contrast, no colocalization with the vacuolar marker was observed for Pmp2GFP-KKTN. We tested whether the observed changes in steady-state localization could be explained by a reduced affinity of the double-mutant COPI coat for the respective signal-containing tails. Binding assays with cytosol obtained from wild-type or double-mutant yeast cells revealed that the mutant COPI complex containing both β-COP* and δ-COP* recognized a KKTE signal at the C terminus of Mst27 (Sandmann et al., 2003) almost as efficiently as the wild-type coat, whereas binding to the last 36 aa of Kir6.2 containing the LRKR signal was strongly reduced (Fig. 2 C).

To address the specificity of the sorting defect (Fig. 2 B) we investigated the steady-state localization of two additional membrane proteins known to be retrieved by COPI. Rer1 is a retrieval receptor for ER membrane proteins that is dynamically localized to the Golgi apparatus by COPI (Sato et al., 2001). Emp47 is a cargo receptor that shuttles between the ER and the Golgi (Schroder et al., 1995; Sato and Nakano, 2002). The C-terminal KTKLL signal of Emp47 is thought to be recognized by the WD40 domain of β′-COP (Eugster et al., 2004). Indirect immunofluorescence and subcellular fractionation on sucrose gradients revealed similar steady-state localization patterns for both Rer1 and Emp47 in wild-type and double-mutant cells (Fig. 3, A and B). The punctate staining pattern is consistent with their accumulation in Golgi sub-compartments. The vacuolar protein carboxypeptidase Y (CPY) was found to float to the light fractions (labeled 1, 2 in Fig. 3 B) of the gradient for both strains, whereas the heavy fractions (labeled 7, 8, 9) of the gradient were enriched in the ER marker Kar2. Detection of Pmp2YFP-NVRNRRK (exposing a weak R-based signal) revealed that the reporter cofractionated with the ER marker Kar2 in the wild-type strain and partially redistributed to the lighter fractions in the strain coexpressing both, β-COP* and δ-COP*. This confirms the mislocalization of this reporter in the double-mutant strain. The most apparent defect in the double-mutant strain consisted of multiple vacuoles as observed by FM4-64 staining (Fig. 2 B) or indirect immunofluorescence of a vacuolar membrane protein (Fig. 3 A).

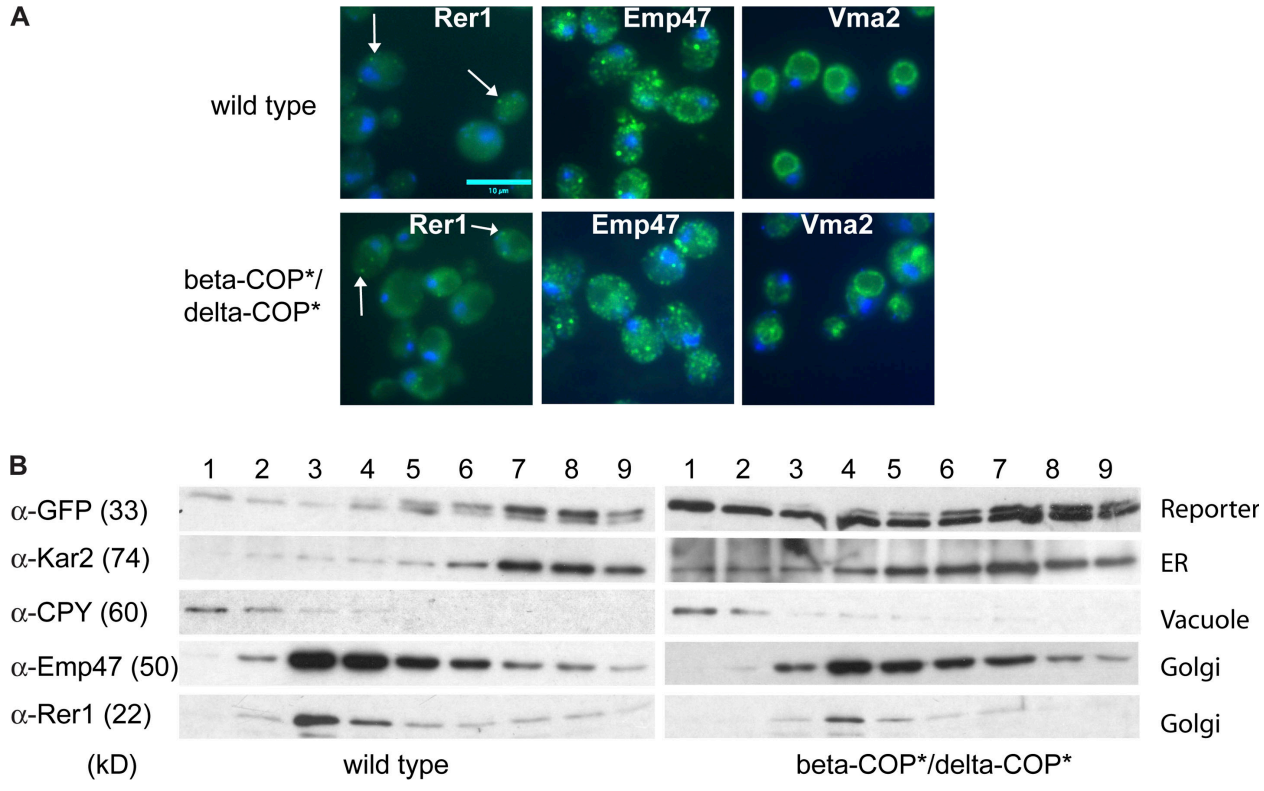

Figure 3. **COPI-dependent retrograde cargo is correctly localized in cells with mutant coatomer supporting the specificity of the sorting defect for R-based signals.** (A) Indirect immunofluorescence of the cells coexpressing β-COP* and δ-COP* (Sec26 ^{334}DLD/NAN and Ret2Δ414-435) or wild-type cells using primary antibodies against the indicated proteins and a fluorescent secondary antibody (depicted in green). Nuclei were stained with DAPI (blue). White arrows mark Rer1-positive puncta. (B) Subcellular fractionation of wild-type or double-mutant yeast cells expressing Pmp2YFP-NVRNRRK. Cells were grown at 25°C. Homogenates were prepared from spheroplasts and loaded on ten-step sucrose gradients (fraction 1: 18% sucrose, fraction 9: 50% sucrose). Membranes were sedimented from the fractions indicated and 5 μg of membrane protein was loaded in each lane followed by Western blotting analysis using the indicated antibodies (the anti-GFP signal reflects the distribution of the Pmp2YFP-NVRNRRK reporter in the gradient, Kar2 is an ER marker, CPY is a vacuolar protein, Emp47 and Rer1 are proteins that depend on COPI for their correct localization to the Golgi).

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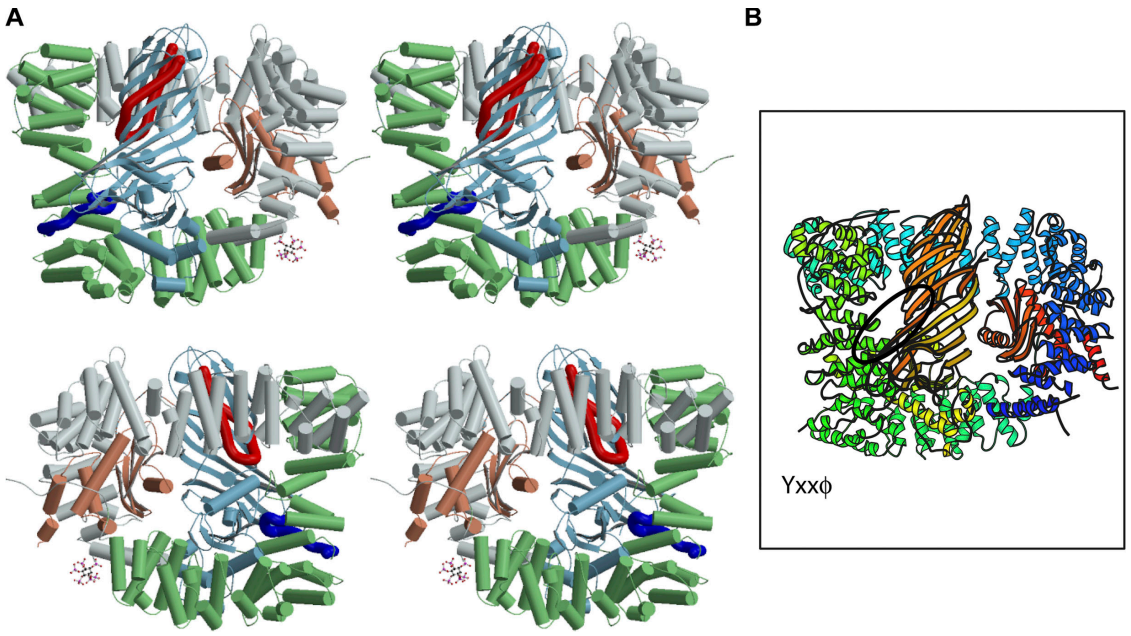

Figure 4. **Position of the identified binding site(s) in a homology model of the COPI trunk domain.** (A) Stereo pairs of two different orientations of the homology model lacking the appendage domains. β-COP is shown in green, γ-COP in gray, δ-COP in light blue and ζ-COP in brown. The mapped, critical region is shown in dark blue (β-COP) and in red (δ-COP). The position of the PI4-P binding site present in the AP-1 structure is indicated by the PI4-P molecule in ball-and-stick representation. (B) Ribbon presentation of the clathrin adaptor 1 core based on the coordinates deposited by Heldwein et al. (2004). The black ellipse indicates the binding site for YXXΦ signals as shown in Fig. 1 A of Heldwein et al. (2004).

We conclude that the COPI coat containing both β-COP* and δ-COP* is specifically incapable of recognizing R-based signals. At the same time this mutant coat is still capable of sorting three other cargo proteins with high fidelity. The two independent or one common binding site(s) formed by β- and δ-COP are necessary and sufficient to recognize R-based peptide sorting motifs. Importantly, the region contributing to the recognition site for R-based signals is highly conserved across eukaryotic species, consistent with the fact that the signals are effective and follow the same consensus in yeast and mammalian cells. The effects of mutating or deleting the critical stretch in either β- or δ-COP were additive, e.g., individual mutations in either β- or δ-COP caused considerable ER exit of the Pmp2YFP reporter exposing a weak R-based signal, whereas the reporter exposing the strong signal remained mostly ER-localized (Fig. 2 A). When both mutations were combined, the less efficient signal was completely inactive as an ER localization motif and reporters presenting the stronger LRKR or KLRRRRI signals accumulated outside the ER. This additive behavior supports the notion that the regions in the two subunits contribute to a common binding site that is completely distinct from the binding site recognizing KKXX signals.

The binding site for R-based signals in the trunk of COPI is in the same region that recognizes YXXΦ signals in adaptor complexes

No high-resolution structure of the COPI coat complex is available, but sequence homology and partial structures suggest that the β-, δ-, γ-, and ζ-COP subcomplex is structurally similar to the clathrin-adaptor core complex, whereas the α-, β'-, and ε-COP subcomplex is thought to be functionally equivalent to clathrin (Schledzewski et al., 1999; Owen et al., 2004; Langer et al., 2007). To better understand the structural relationship between the stretches identified in β- and δ-COP, we built a homology model of the adaptor-like COPI subcomplex containing β-, δ-, γ-, and ζ-COP based on the crystal structure of the clathrin adaptor 1 core (Heldwein et al., 2004) (Fig. 4 A; compare the original structure shown as Fig. 4 B for reference). Our homology model is consistent with the idea that the relevant regions of β- and δ-COP come into close proximity and with the concept of one binding site for R-based signals at the subunit interface. Strikingly, this binding site in the adaptor-like trunk is structurally comparable to the site where YXXΦ endocytic motifs are recognized by clathrin adaptors (Fig. 4 B, compare red and blue regions in Fig. 4 A to black ellipse). This suggests that the general architecture of the trunk structure has evolved to accommodate completely different cargo-sorting signals. It will be interesting to test how the recognition of R-based signals is coupled to the sorting of ion channel and receptor subunits.

Materials and methods

Construction of plasmids

Molecular cloning followed standard procedures as described in Ausubel et al. (1997). All plasmids are listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200704142/DC1). All constructs were verified by sequencing. Pmp2GFP and YFP fusions were as described in Michelsen et al. (2006). Two additional constructs were prepared where Pmp2 was fused to YFP followed by a unique NotI site. The NotI site encoded three alanines linking YFP and the tail. Oligonucleotides coding for the different tails were cloned into NotI and XhoI sites, resulting in constructs pR889 –AAATLAS**KLRRRRI**SLS and pR890 –AAATLAS**NVRNRRK**SLS (compare Table S1).

The GST fusions are derivatives of the GST-MST27-KKXX construct (Sandmann et al., 2003). The construct is a fusion of the C-terminal tail of

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Mst27 (codons 159–234 including stop codon) to GST. The different R-based signals were introduced as oligonucleotides using a ClaI site (codon 226 of *MST27*) and an XhoI site present in the pGex6P vector. The sequences of the different tails are as follows in the indicated constructs (compare Table S1): pO732 -^{227}DALL**KKTE** and pF291 -^{227}DLLDALTLASSRGP**LRKR**SVAVAKAKPKFSISPDSLSGSRSHHHHHH. Bold-print letters indicate the peptide-sorting motif.

Construction of *ret2* and *sec26* mutant strains

The yeast strains used in this study are listed in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200704142/DC1). A diploid heterozygous *ret2Δ* strain (Y25865) was transformed with an *URA3*-containing *CEN* plasmid (pQ849) carrying the temperature-sensitive *ret2-1* allele. The strains were then sporulated and spores were selected for their capacity to grow on geneticin (the *ret2Δ* genotype was marked with a *kanMX4* selection marker in strain Y25865) and for 5-fluororotic acid (5′FOA) sensitivity to ensure loss of the wild-type *RET2* allele present in the diploid strain. Plasmids carrying either wild-type *RET2* (Y25865-KM1) or *RET2Δ*414-435 (Y25865-KM2) under the control of the *RET2* promoter were then integrated into the *HIS3* locus of the resulting haploid strains. These strains were selected on 5-FOA for loss of the *ret2-1*-carrying *URA3* plasmid. In Y25865-KM2, the presence of only the altered *RET2* variant after gene swapping was confirmed by the polymerase chain reaction and by Western blot analysis of total cell lysates using an anti-COPI antiserum.

To test whether different mutant *SEC26* alleles could substitute for the wild-type gene, we used a strain (RDY122) with an integrated copy of *SEC26* under the control of the *GAL1* promoter (Duden et al., 1994). The strain was transformed with a *CEN* plasmid carrying the respective variant of *SEC26* (*SEC26* ^{334}DLD/NAN or ^{325}DILR/NAAA or Δ318-338) under the control of a *MET25* promoter (p894-p897) and a *LEU2* marker. Serial dilutions of individual transformants were spotted on selective medium lacking leucine and containing glucose to suppress transcription from the *GAL1* promoter. All transformants that had received *SEC26* wild- type or mutant alleles but not those that had received only the empty plasmid were able to grow in the presence of glucose indicating that all Sec26-derived proteins were functional components of coatomer.

Strain Y25865-KM2 was transformed with a *URA3*-marked 2μ plasmid carrying the wild-type *SEC26* gene. Next, a *natMX4* cassette disrupting the coding region of *SEC26* was introduced by homologous recombination and the resulting transformants were selected for growth on nourseothricin-containing medium. This strain was then transformed with a *LEU2*-marked *CEN* plasmid carrying the different *SEC26* alleles (*SEC26* ^{334}DLD/NAN or ^{325}DILR/NAAA or Δ318-338). Transformants were selected on 5′FOA-containing medium to select against the plasmid carrying the wild-type *SEC26* gene yielding strains Y25865-KM3 and Y25865-KM4.

Large-scale transformation and microscopy

Large-scale transformations were performed in 96-well plates using the lithium acetate method (Ausubel et al., 1997) and transformants were plated on synthetic complete (SC) medium lacking uracil or leucine. Liquid cultures were inoculated in 96-well plates containing SC medium lacking uracil or leucine and viewed by epifluorescence after overnight growth at 30°C. For screening of the *tetO7* promoter allele library 40 μg/ml doxycycline was added to the medium during the overnight growth period before microscopy. Automated microscopy of the two yeast libraries was performed on an Olympus ScanR screening system (40 × 0.95 objective) and 96-well glass-bottom plates (MMI).

Yeast two-hybrid analysis

Double transformants of strain AH109 containing Gal4-BD and -AD plasmid were selected on SC medium lacking tryptophane and leucine. In contrast to Zerangue et al. (2001), we replaced the auto-activating Gal4-BD fusions of bovine β- and ζ-COP with the respective fusions to the corresponding yeast COP subunits (encoded by *SEC26* and *RET3*) and performed the two-hybrid assays at low stringency (e.g., dilution series of double transformants grown in SD lacking tryptophane and leucine were spotted on SD medium lacking tryptophane, leucine, and histidine to test for the activation of the *HIS3* reporter gene). The plasmid encoding the Gal4-BD fusion to Ret3 proved toxic to the yeast and was thus omitted from further experiments. The deletion analysis was performed on bovine δ-COP and yeast β-COP.

Preparation of cell lysates

Crude cell extracts were prepared essentially as described by Schultz (1999). In brief, yeast cells were grown to logarithmic phase, harvested, and resuspended in cold wash buffer (20 mM Hepes, pH 7.4, 0.7 M sorbitol, and 1 mM PMSF). Cells in wash buffer were transferred to a syringe and pelleted for 5 min at 2,000 rpm. Supernatant was removed and the paste was extruded from the syringe into liquid nitrogen. Frozen cells were ruptured with a pestle and mortar filled with liquid nitrogen. The resulting white powder was transferred to a reaction tube and an equal volume of binding buffer (20 mM Hepes, pH 6.8, 2% glycerol, 150 mM KAc, 5 mM $Mg(Ac)_2$, 1 mM EDTA, 1 mM DTT, and 0.1% Triton-X 100 containing protease inhibitor mix: 2.5 μg/ml leupeptin, 1.5 μg/ml antipain, 0.5 μg/ml chymostatin, and 1 μg/ml pepstatin A) was added. After thawing, the crude extract was cleared for 5 min at 2,000 rpm to remove unbroken cells.

Purification of GST fusion proteins

GST fusion proteins were induced in BL21 (DE3 star) carrying pRosetta by adding 0.2 mM IPTG for 2 h at 37°C. Cells were harvested and sonified in breaking buffer (20 mM Hepes, pH 6.8, 2% glycerol, 150 mM KAc, 5 mM $Mg(Ac)_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1× Complete protease inhibitor cocktail [Roche]). The lysate was cleared for 5 min at 2,000 rpm. The resulting supernatant was subjected to ultra-centrifugation for 30 min at 100,000 *g*. The GST fusion proteins were purified from the supernatant by incubating with GSH-agarose. The agarose column was washed with breaking buffer and proteins were eluted by adding elution buffer (20 mM Hepes, pH 9.5, 10 mM GSH, 5% glycerol, 150 mM KAc, 5 mM $Mg(Ac)_2$, 1 mM EDTA, and 1 mM DTT). For purification of the GST fusion protein with –LRKR-HexaHis C-terminus a Protino-2000 Ni-column (Macherey) was used as per the manufacturer's instructions. Appropriate fractions were dialyzed overnight in binding buffer and 0.5 μg dialyzed protein bound to 1 μl GSH-agarose (Sigma-Aldrich).

Binding assay

For binding experiments, crude extract was cleared of all insoluble material for 30 min at 100,000 *g* and the supernatant was used for binding experiments. 100 μg total protein in 500 μl binding buffer (containing protease inhibitors, see above) was used for a binding assay with 2.5 μg bait on 5 μl GSH-agarose and incubated 2 h at 4°C. Beads were washed five times in binding buffer. Bound proteins were eluted with 1× SDS loading buffer containing 1 mM DTT, resolved by SDS PAGE, and detected by Western blotting using rabbit anti-COPI and rabbit anti-GST (Sigma-Aldrich) primary antibodies and an anti–rabbit AlexaFluor 680 (Invitrogen) secondary antibody. Blots were scanned (700 nm) and quantified with the Li-COR Odyssey system.

Subcellular fractionation on sucrose gradients

Separation of organelles was performed according to Nass and Rao (1998). In brief, cells were grown to mid-logarithmic phase, converted to spheroplasts, and lysed by douncing in a hypotonic buffer (0.3 M sorbitol and 50 mM triethanol amine, pH 8.9). The homogenate was layered on freshly prepared ten-step sucrose gradients and centrifuged for 12 h at 23,500 rpm in an SW28 rotor (Beckman Coulter). Membranes from the different fractions were pelleted by centrifugation (SW28, for 30 min at 23,500 rpm).

Immunostaining of yeast cells

Cells were grown to early logarithmic phase and fixed at room temperature in freshly prepared fixative for at least 8 h and processed as described by Roberts et al. (1991). In brief, spheroblasts were permeabilized by a short incubation in 1% SDS in 1.2 M sorbitol and then stained with the indicated primary and Alexa594-conjugated secondary antibodies (Invitrogen).

Fluorescence microscopy

Microscopy was performed using a microscope (DM IRE2; Leica) controlled by OpenLab software (Improvision) with a 100×/1.4–0.7 HCX PL APO CS oil immersion objective. Images were captured by an Orca-ER CCD camera (Hamamatsu) with excitation at 470/40 nm and emission at 525/50 nm (GFP, YFP), or excitation at 510/40 nm and emission at 610 nm/longpass (FM4-64, Alexa594). All images shown are representative of several independent experiments. Steady-state staining with FM4-64 was performed in SC medium for 1 h at room temperature. For live-cell imaging, yeast were incubated in SC medium at room temperature. Fixed yeast cells were mounted in ProLong Gold AntiFade reagent with DAPI (Invitrogen). Live cell images of transiently transfected HeLa cells were acquired using a 63×/1.4–1.6 HCX PL APO oil immersion objective in L-15 medium (Leibovitz; Sigma-Aldrich) at 37°C. Images were transferred to Adobe Photoshop CS2 for slight gamma adjustments.

Comparative modeling of yeast COPI

A comparative three-dimensional model for the tetrameric subcomplex of yeast COPI was built based on the Crystal Structure of the clathrin adaptor

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protein core AP-1 (PDB: 1w63; Heldwein et al., 2004). Template identification for all four chains of the yeast COPI trunk lacking the appendage domains was performed by searching the PDB database (Berman et al., 2000) for suitable template structures in SwissModel Workspace (Arnold et al., 2006). Target-template alignments were generated by structure-guided multiple sequence alignment using 3DCoffee (O'Sullivan et al., 2004) for aligning γ-COP with template chain 1w63:A, β-COP with 1w63:B, and ζ-COP with 1w63:S. The alignment between δ-COP with 1w63:M was generated by HMM-HMM alignment (Soding, 2005). Model coordinates for the tetrameric complex were generated using satisfaction of spatial restraints (Sali and Blundell, 1993) after visual assessment of placements of insertions and deletions in the alignments.

Online supplemental material

Figure S1 shows microscopic images of selected hits in the reverse genetic screen expressing Pmp2YFP-LRKR and Pmp2YFP-KKTN. Figure S2 shows the localization of different Pmp2-GFP reporters in strains lacking ε-COP or expressing mutant α-COP. Figure S3 shows the steady-state localization of CD4GFP reporters exposing a very strong and a weak R-based signal in HeLa cells. Table S1 provides information about the plasmids and Table S2 about the yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704142/DC1.

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