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Dsl1p, an Essential Component of the Golgi-Endoplasmic Reticulum Retrieval System in Yeast, Uses the Same Sequence Motif to Interact with Different Subunits of the COPI Vesicle Coat*[S]

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Dsl1p is required for Golgi-endoplasmic reticulum (ER) retrograde transport in yeast. It interacts with the ER resident protein Tip20p and with δ-COP, a subunit of coatomer, the coat complex of COPI vesicles. To test the significance of these interactions, we mapped the different binding sites and created mutant versions of Dsl1p and δ-COP, which are unable to bind directly to each other. Three domains were identified in Dsl1p: a Tip20p binding region within the N-terminal 200 residues, a highly acidic region in the center of Dsl1p containing crucial tryptophan residues that is required for binding to δ-COP and essential for viability, and an evolutionarily well conserved domain at the C terminus. Most importantly, Dsl1p uses the same central acidic domain to interact not only with δ-COP but also with α-COP. Strong interaction with α-COP requires the presence of comparable amounts of ε-COP or β′-COP. Thus, the binding characteristics of Dsl1p resemble those of many accessory factors of the clathrin coat. They interact with different layers of the vesicle coat by using tandemly arranged sequence motifs, some of which have dual specificity.

The structural integrity of membrane-bound organelles requires the recycling of lipids and proteins. Between Golgi and the endoplasmic reticulum (ER)[1] this retrograde transport is mediated by COPI-coated vesicles (1). The COPI coat from yeast and mammals consists of seven COP proteins (α-, β′-, β-, γ-, δ-, ε-, and ζ-COP = coatomer) and the small GTPase ARF1 (2, 3). Sorting of cargo proteins to COPI vesicles can be achieved by direct binding of cargo molecules to coatomer (4–8). This binding is often mediated by short sequence motifs displayed by cargo molecules (6, 9). The efficient sorting of cargo into COPI vesicles depends on GTP hydrolysis by ARF1 facilitated by a specific GTPase-activating proteins (10).

After the uncoating, vesicles are ready to fuse with their specific target membrane (11). Fusion events rely on specific attachment reactions to guarantee that only appropriate membranes can mix. The membrane attachment itself comprises two steps, tethering and docking (12). Both steps involve different sets of proteins. Tethering factors are peripherally membrane-associated protein complexes consisting of up to 10 different subunits, which share little sequence similarity. So far, seven different tethering complexes required for at least five different transport steps were characterized in yeast (13–20). In some cases, mammalian counterparts of yeast tethering factors were identified (reviewed in Ref. 21).

The subsequent docking stage involves specific sets of membrane-anchored proteins, the so-called SNARE proteins (22, 23). In contrast to the tethering factors, all known SNARE proteins are members of either of three protein families: the syntaxins, the synaptobrevins or VAMPs and the SNAP-25 family members. To induce membrane fusion, SNARE proteins from apposed membranes must interact in *trans*. The SNARE or SNARE-like proteins involved in fusion at the ER are Ufe1p, Sec22p, Sec20p, and Use1p (24–27). Tip20p as a Sec20p-interacting protein is also essential for retrograde transport (25, 28–30). So far no evidence for an involvement of a Ypt/Rab-like GTPase or specific tethering factors has been provided.

Recently, we and others identified a new coatomer-interacting factor, Dsl1p, which is essential for retrieval of proteins from Golgi to ER (31–33). Dsl1p also interacts with Tip20p (33, 34). This suggests that Dsl1p may play an important role in targeting COPI vesicles to the ER membrane.

In this work we show that Dsl1p and δ-COP bind each other directly. We mapped the binding sites in both molecules, and mutants were created to investigate the significance of the Dsl1p/δ-COP interaction *in vivo*. Although mutations in Dsl1p that block the binding to δ-COP *in vitro* were lethal, δ-COP (*ret2*) mutants carrying deletions in the putative Dsl1p binding domain remain viable. The fact that these mutant proteins retained some Dsl1p binding activity prompted us to reinvestigate the Dsl1p/coatomer interaction. We found that Dsl1p can also make contacts with the putative outer layer of the coatomer complex. Dsl1p uses overlapping sequence motifs to interact with δ-COP and α-COP. In this respect Dsl1p resembles many accessory factors or cargo-specific adaptors known to be involved in the formation of clathrin-coated vesicles.

EXPERIMENTAL PROCEDURES

Bacterial and Yeast Strains—Cloning experiments were performed using the *Escherichia coli* strain DH5α. Expression of either His_6-tagged or GST fusion proteins was performed using protease-deficient strains BL21DE3 or C41DE3 as described previously (32). *Saccharomyces cerevisiae* strains used are listed in Table I. Cells were grown in yeast extract-peptone-or synthetic minimal medium both containing glucose (2%) and supplemented as necessary with 20 mg/liter tryptophan, histidine, adenine, uracil, or 30 mg/liter leucine or lysine. 5-Fluoroorotic acid (5-FOA) plates were prepared as synthetic minimal me-

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[S] The on-line version of this article (available at http://www.jbc.org) contains discussion of identification of DSL1-related sequences (Supplemental Figs. 1–3, text, and Supplemental Refs. 1–8).

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[1] The abbreviations used are: ER, endoplasmic reticulum; ARF, ADP ribosylation factor; COP, coatomer protein; GST, glutathione *S*-transferase; SNARE, soluble NSF attachment protein receptor; aa, amino acid(s); 5-FOA, 5-fluoroorotic acid; HA, hemagglutinin.

TABLE I
Yeast strains

Strain	Genotype	Source
MSUC-3B	*MATα, ade2, ura3, leu2, his3*	This laboratory
PJ-694A	*MAT**a**, ura3–52, leu2–3,112, trp1–901, his3–200, ga14*Δ, *gal80*Δ, *LYS2*::*GAL1-HIS3*, *GAL2-ADE2*, *met2*::*Gal7–lacZ*	P. James
S21P4–9A	*MAT**a**, leu2, ura3, his3, pep4*::*HIS3, sec21–1*	This laboratory
S21P4/8–5C	*MATα, leu2, ura3, lys2, trp1, his3, ret2–1*	This laboratory
Y21186	*MAT**a**/**α**, ura3*Δ*0/ura3*Δ*0, leu2*Δ*0/leu2*Δ*0, his3*Δ*1/his3*Δ*1, lys2*Δ*0/LYS2, MET15/met15*Δ*0, YNL258c*::*Kan*MX/*YNL258c*	EUROSCARF
YUA1–9C	*MATα, ade2, ura3, leu2, his3, lys2, dsl1–22*	This study
YUA12–1A	*MAT**a**, ura3, leu2, his3, dsl1*::*Kan*MX, containing pUA89 (GFP-*DSL1, URA3*)	This study
BY4742	*MATα, ura3, leu2, his3, lys2*	EUROSCARF
Y11469	*MATα, sec28*Δ (*YIL076w*::*Kan*MX4), *ura3, leu2, his3, lys2*	EUROSCARF
Y25865	*MAT**a**/**α**, ret2*Δ (*YFR051c*::*Kan*MX4)/*RET2, ura3/ura3, leu2/leu2, his3/his3, lys2/LYS2, MET15/met15*	EUROSCARF
R2P4–5C	*MAT**a**, ret2–1, ura3, leu2, his3, trp1, lys2*	This study

dium containing 0.1% 5-FOA. Yeast transformations were performed as described previously (35). Standard techniques were used for complementation analysis, sporulation, and the analysis of tetrads (36). Deletions were introduced into the δ-COP encoding *RET2* gene on a centromeric vector. A heterozygous *ret2* deletion strain (Y25865) was transformed with these constructs, and tetrads were dissected to obtain spores expressing only the mutated *RET2* genes.

Cloning and Plasmids—Plasmids used in this work are listed in Table II. Recombinant wild type and mutant *DSL1* as well as fragments derived from it were expressed as GST fusions. The *DSL1* sequences were inserted into vector pGEX-TT (pGEX-2T with modified polylinker region; Amersham Biosciences). The fragments inserted into this vector were created by PCR. pUA73 (pRS315-*DSL1*) served as template and *Pfu* DNA polymerase (Stratagene) was used to amplify the fragments. The primers we used contained XmaI (5′) and XbaI (3′) sites allowing in frame insertion of the coding segments into pGEX-TT. Alanine substitutions were generated by site-directed mutagenesis using the QuikChange kit (Stratagene). *DSL1* fragments L1–L6 were generated by annealing of oligonucleotides with flanking BamHI (5′) and HindIII sites (3′) and integrated into the pGEX-TT, which had been digested with the BamHI and HindIII. Full-length Ret2p/δ-COP and its truncations were expressed as N-terminal His_6 fusions using pQE30 (Qiagen). The primers used for the PCR introduced BamHI (5′) and HindIII (3′) sites flanking the PCR product. Yep24-*SEC21* and pRS426-*RET3* were tested for complementation as well as multicopy suppression (YEp24-*SEC21*) of the corresponding mutations to prove that they are functional.

Yeast Two-hybrid Analysis—The yeast two-hybrid assay was performed as described previously (37) using different Dsl1p versions fused to the Gal4p-DNA binding domain as bait and a Ret2p fragment (bases 466–1641) fused to the Gal4p activation domain as prey (isolate 2, Ref. 33).

Purification of Recombinant Proteins and Affinity-binding Assay—*E. coli* strains expressing His_6 fusion proteins were lysed and proteins were solubilized in phosphate buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 0.5% Triton X-100, protease inhibitor mix (Roche Molecular Biochemicals)). His_6-tagged proteins were immobilized on nickel-nitrilotriacetic acid-agarose, washed five times with 10 volumes of phosphate buffer, and eluted using 250 mM imidazole. *E. coli* strains expressing GST fusion proteins were lysed, and proteins were solubilized in buffer B88 (20 mM Hepes, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 0.5% Triton X-100, containing EDTA-free protease inhibitor mix). GST fusion proteins were immobilized on glutathione-Sepharose 4B and washed five times with 10 volumes of lysis buffer. Bound proteins were eluted from the Sepharose beads with 10 mM glutathione and analyzed by SDS-PAGE. For pull-down experiments, equal amounts of fusion proteins were loaded on glutathione-Sepharose 4B beads again and incubated either with His_6-tagged proteins or with the 8,000 × *g* supernatant of yeast cell lysates at 4 °C for 2–4 h. For the experiments shown in Fig. 6 and the *lower panel* in Fig. 7*A*, incubation was done 1.5 h at room temperature. The beads were washed five times with buffer B88, and proteins were separated by SDS-PAGE followed by immunoblot analysis or Coomassie staining (Figs. 1, 2*B*, 4, *B* and *C*, and 7). To obtain extracts yeast cells were broken with glass beads using buffer B88 (see above). The LumiImager software (Roche Molecular Biochemicals) was used to quantify chemiluminescence signals of immunoblots.

Peptide Binding Experiments—14-mer peptides containing a N-terminal cysteine residue for the later coupling step were synthesized by the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) method on a Biosearch 9050 Pep-synthesizer (Milligen). Removal of protecting groups and cleavage of peptides from the resin were achieved by treatment with a solution of 90% trifluoroacetic acid, 5% water, 2.5% phenol, and 2.5% 1,2-ethanedithiol (all from Sigma). Peptides were immobilized on agarose gel using the SulfoLink kit (Pierce) following the instructions of the manufacturer. Immobilized peptides were incubated at room temperature with indicated GST fusion proteins or *E. coli* lysate in 1 ml of B88 (see above) containing 1% Triton X-100 for 2 h. After washing thoroughly, bound proteins were eluted by 100 mM glycine, pH 2.4. 1-ml fractions of both washing and elution steps were collected; elution fractions were neutralized with 50 μl of 1.5 M Tris (pH 8.8) solution and subsequently analyzed by SDS-PAGE and Coomassie staining.

Antibodies—Polyclonal anti-Dsl1p antibodies were a gift from M. Gerard Waters (Princeton University, Princeton, NJ), the anti δ-COP antibodies were obtained from Stephan Schröder-Köhne (BioMedTec, Wuerzburg, Germany), and the polyclonal anti-coatomer and the monoclonal anti-HDEL antibodies were gifts from Rainer Duden and Mike Lewis (Cambridge University, Cambridge, United Kingdom). Anti-coatomer antibody (38) cross-reacted with GST-Dsl1p. Therefore, 10 μl of anti-coatomer serum were incubated with 80 μg of GST-Dsl1p coupled to GSH-Sepharose to remove unspecific antibodies. Horseradish peroxidase-coupled secondary anti-rabbit or anti-mouse antibodies were purchased from Jackson Laboratories.

Analysis of BiP/Kar2p Secretion—Early log phase *dsl1* deletion strains expressing either wild type *DSL1* or *dsl1* mutants from single copy plasmids were spotted onto YPD plates. Plates were overlaid with nitrocellulose and after 12-h incubation at 33 °C filter were removed and analyzed for Kar2p secretion by immunoblotting using a Kar2p-specific antibody and the ECL detection system (Amersham Biosciences).

RESULTS

Dsl1p Binds to δ-COP via Its Central Acidic Domain—In our previous work we detected binding of coatomer to Dsl1p by using a GST-Dsl1p fusion construct expressed in *E. coli*. Consistent with this, Reilly *et al.* (33) identified the coatomer subunit δ-COP, as a Dsl1p-interacting protein in a yeast two-hybrid screen. To test whether this interaction can be reproduced *in vitro*, we repeated the GST-Dsl1p pull-down experiment (32) using extracts from yeast cells overproducing either δ-COP or α-COP and β′-COP as control (4, 38). As shown in Fig. 1, only the overexpression of the δ-COP-encoding *RET2* gene increased the amount of protein retained on GST-Dsl1p beads significantly. The protein was detectable by Coomassie staining. Immunoblot analysis was performed to confirm that this band in fact represents δ-COP (data not shown).

A closer look at the Dsl1p primary structure revealed the presence of a δ-COP binding motif (δL) that has been characterized before by Cosson *et al.* (7). The spacing of two tryptophans and the composition of surrounding residues resemble that of the δL motif. The di-aromatic motif in Dsl1p is part of a glutamate-rich domain in the center of the protein (-E-E-E-E-N-A-**W**-E-D-A-**W**-A- = W*XXX*W motif). The original δL motif is at the C terminus of the hypothetical protein YOR112w (-D-D-

TABLE II
Plasmids

Plasmid name	Description	Source
pBR2	Gal4p-DNA binding domain-Dsl1p, *URA3*	M. G. Waters
pGAD-C1	Gal4p-activation domain, *LEU2*	P. James
pGBDU	Gal4p-DNA binding domain, *URA3*	P. James
pGWB502	Gal4p-AD-Ret2p (bp 466–1641), *LEU2*	M. G. Waters
pRS323-*RET2*	*RET2* (wild type or deletion mutants), *HIS3, 2 μm*	This laboratory
p22-*RET2*	*RET2* (wild type or deletion mutants), *URA3, CEN*	F. Letourneur
pRS323-*RET1*	*RET1 (COP1), HIS3, 2 μm*	This Laboratory
pRS323-*RET1-S28*	*RET1 (COP1), SEC28, HIS3, 2 μm*	This study
YEp24-*SEC21*	*SEC21, LEU2, 2μm*	R. Duden
pRS426-*RET3*	*RET3, URA3, 2μm*	B. A. Kraynack
YEp24-*SEC27*	*SEC27, URA3, 2 μm*	R. Duden
YEp24-*SEC28*	*SEC28, URA3, 2 μm*	R. Duden
pTM2	*TIP20*-myc, 2 μm, URA3	M. Lewis
pUA73	pRS315-*DSL1, LEU2, CEN6/ARS4*	This laboratory
pUA81	pRS325-*DSL1, LEU2, 2 μm*	This laboratory
pUA86	pRS315-*dsl1–22, LEU2, CEN6/ARS4*	This laboratory
pUA89	pUG36-*DSL1, URA3*	This laboratory
pUA93	pGEX-TT-*DSL1*	This laboratory
pUA135, 136, 137	pGEX-TT-L1(W*XXX*W), L2(A*XXX*W), L3(W*XXX*A)	This study
pUA141	pRS315-*DSL1*^{W455A}, *LEU2, CEN6/ARS4*	This study
pUA143	pGEX-TT-*DSL1*-Δc (aa 1–445)	This study
pUA151	pQE30-*RET2*	This study
pUA152	pQE30-*ret2*Δc (aa 1–400)	This study
pUA157	pGEX-TT-*DSL1*$^{W455A/W459A}$	This study
pUA162, 162, 163	pGEX-TT-*DSL1*-aa 1–200, -aa 151–350, -aa 301–449	This study
pUA165	pGEX-TT-*DSL1*-ΔN (aa 461–754)	This study
pUA168	pGEX-TT-*DSL1*$^{W413A/W455A}$	This study
pUA172, 175, 173	pGEX-TT-L4 (W*X*W), -L5(A*X*W), -L6(A*XW XXX*A)	This study
pUA180	pQE30-*ret2*ΔN(aa 281–546)	This study
pUA183	pRS315-*DSL1*$^{W413A/W455A}$, *LEU2, CEN6/ARS4*	This study
pUA189	pRS315-*DSL1*^{W413A}, *LEU2, CEN6/ARS4*	This study
pUA190	pGEX-TT-*DSL1*^{W413A}	This study
pUA200	pQE30-*RET2*-aa 191–400	This study
pUA222	Gal4p-DNA binding domain-*DSL1*^{W413A}, *URA3*	This study
pUA223	Gal4p-DNA binding domain-*DSL1*^{W455A}, *URA3*	This study
pUA224	Gal4p-DNA binding domain-*DSL1*$^{W413A/W455A}$, *URA3*	This study

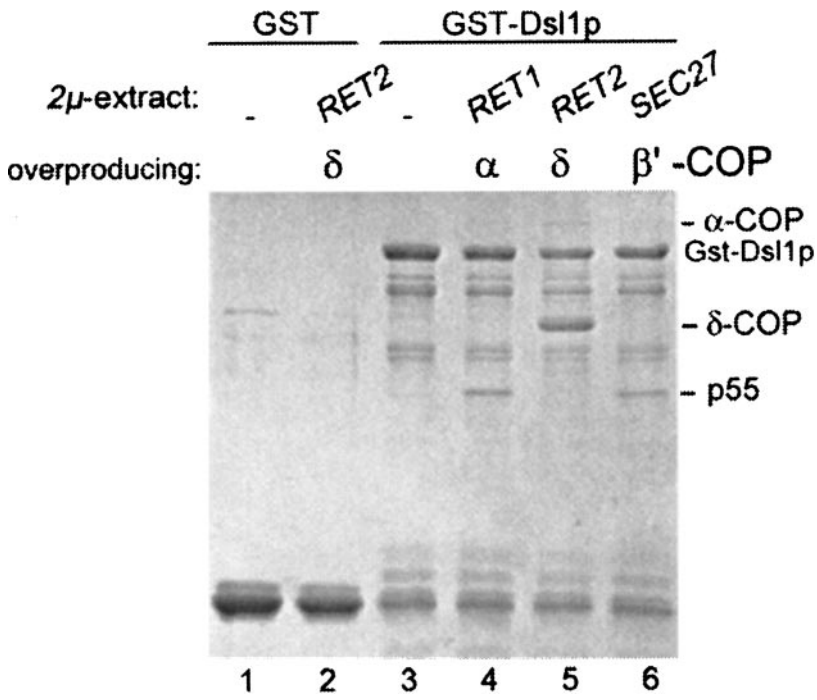


FIG. 1. **δ-COP (= Ret2p) binds to GST-Dsl1p.** Proteins from detergent-lysed yeast cells overexpressing either *RET1* (α-COP), *RET2* (δ-COP), or *SEC27* (β′-COP) from 2-μm vectors were incubated at 4 °C for 2 h with GST alone or GST-Dsl1p purified from *E. coli* and immobilized on glutathione-Sepharose 4B. Beads were washed five times, and the proteins retained were analyzed by SDS-PAGE followed by Coomassie staining. The positions of the α- and δ-COP subunits and additional bound proteins (p55) are indicated.

D-G-D-S-D-S-**W**-D-T-N-**W>**) (Ref. 7). Fig. 2*B* shows that residues 444–459 of Dsl1p containing the δL-like W*XXX*W motif from Dsl1p was able to retain of δ-COP from yeast extracts when fused to GST and immobilized on glutathione Sepharose beads. As in case of the δL-like motif (7), alanine substitution of either of the two tryptophan residues (W455A or W459A = A*XXX*W or W*XXX*A; Fig. 2) resulted in loss of binding to δ-COP. To assess the relevance of these residues, we introduced these mutations into full-length Dsl1p fused to GST and repeated the binding experiments. Surprisingly, even the mutant protein bearing alanine substitutions at both positions (GST-Dsl1$^{W455A/W459A}$ = A*XXX*A) was able to bind coatomer from yeast extract at wild type levels (see also below). This prompted us to examine Dsl1p for additional δ-COP binding motifs. A

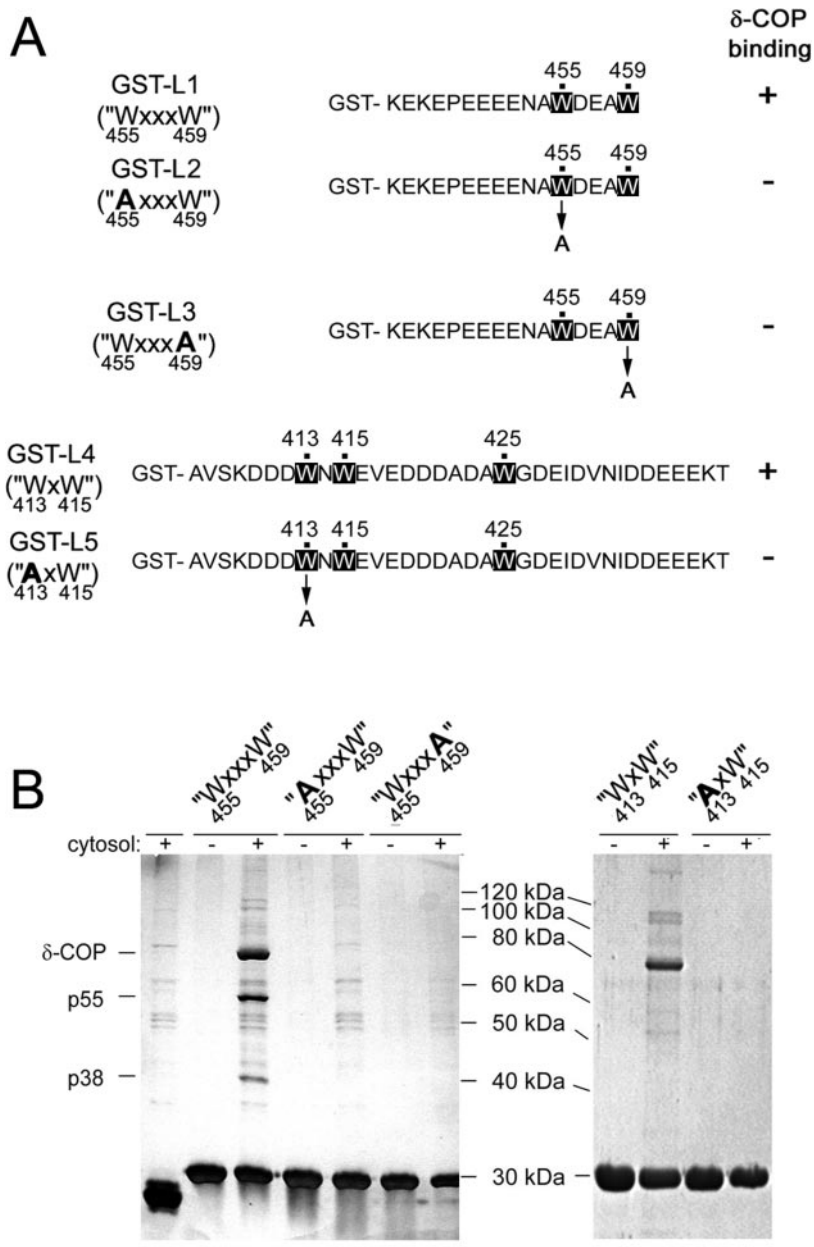


FIG. 2. **Tryptophan residues within an acidic region of Dsl1p are important for the binding to δ-COP.** *A*, GST fusions used for the binding experiments shown in *B*. L1–L3 contain residues 444–459 of Dsl1p fused to GST; L4 and L5 represent wild type and mutant Dsl1p fragments spanning the region from amino acids 406–440, respectively. The fusion proteins carry alanine replacements of tryptophan residues as indicated (L2 = A*XXX*W, L3 = W*XXX*A, L5 = A*X*W; see text). *B*, result of binding experiments using GST-L1 through GST-L5. GST fusion proteins L1–L5 were loaded on glutathione-Sepharose 4B, incubated with extract of δ-COP-overexpressing cells, washed thoroughly (for details see "Experimental Procedures"), and analyzed for bound proteins by SDS-PAGE and Coomassie staining. The δ-COP subunit and proteins retained unspecifically are indicated.

series of fragments derived from Dsl1p were fused to GST and analyzed for their ability to bind δ-COP. The C-terminal part of Dsl1p (aa 461–754) was not able to bind significant amounts of δ-COP. In contrast, the N-terminal portion (aa 1–445) upstream of δL-like W*XXX*W motif bound δ-COP very efficiently. Next we analyzed three overlapping fragments derived from the N-terminal part of Dsl1p to localize the binding region more precisely. Only the fragment spanning residues 301–449 of Dsl1p bound to δ-COP (see also data presented in Fig. 7*A*). At its C terminus this fragment contained an aspartic acid-rich region, which lies adjacent to the glutamic acid-rich δL motif (aa 444–459) analyzed above. This region also contains two closely positioned tryptophan residues (residue 413 and 415; -D-D-D-**W**-N-**W**-E- = W*X*W motif) and a third residue at position 425. To test the functional significance of the W*X*W motif, we fused the aspartic acid-rich domain (aa 406–440) to GST and analyzed this chimeric protein for interaction with δ-COP. The binding efficiency was similar to that observed with the δL-like W*XXX*W-containing fragment (Fig. 2*B*). The binding specificity was higher because no significant amounts of unspecific proteins (p38 and p55) were retained on the beads. A single Trp → Ala substitution of tryptophan 413 (A*X*W) completely abolished the interaction of this Dsl1p fragment to δ-COP (Fig. 2). In summary, two di-aromatic motifs, W*X*W starting at position 413 and W*XXX*W at position 455, contribute to the binding of Dsl1p to coatomer.

To access the relative importance of the W*X*W and W*XXX*W motifs, single and double Trp → Ala substitutions (*dsl1*^{W413A}, *dsl1*^{W455A}, *dsl1*$^{W413A/W455A}$) were introduced into full-length GST-Dsl1p. As mentioned above already, the W455A single substitution (A*XXX*W) did not significantly reduce the extent of binding to δ-COP (Fig. 3*A*). In contrast, the *dsl1*^{W413A} mutant protein (A*X*W) showed a strongly reduced binding compared with the wild type. The double mutant (A*X*W . . . A*XXX*W) had the strongest effect and showed only negligible δ-COP binding *in vitro*. For this experiment, extracts of a δ-COP-overproducing strain had been added to the different GST fusion proteins.

We confirmed these results by yeast two-hybrid analysis (37). The C-terminal part of the δ-COP-encoding *RET2* gene (starting at codon 156 residue, isolate 2) (33) fused to the sequence of the Gal4p activation domain was used as prey. The different *DSL1* versions, wild type or Trp → Ala substitutions,

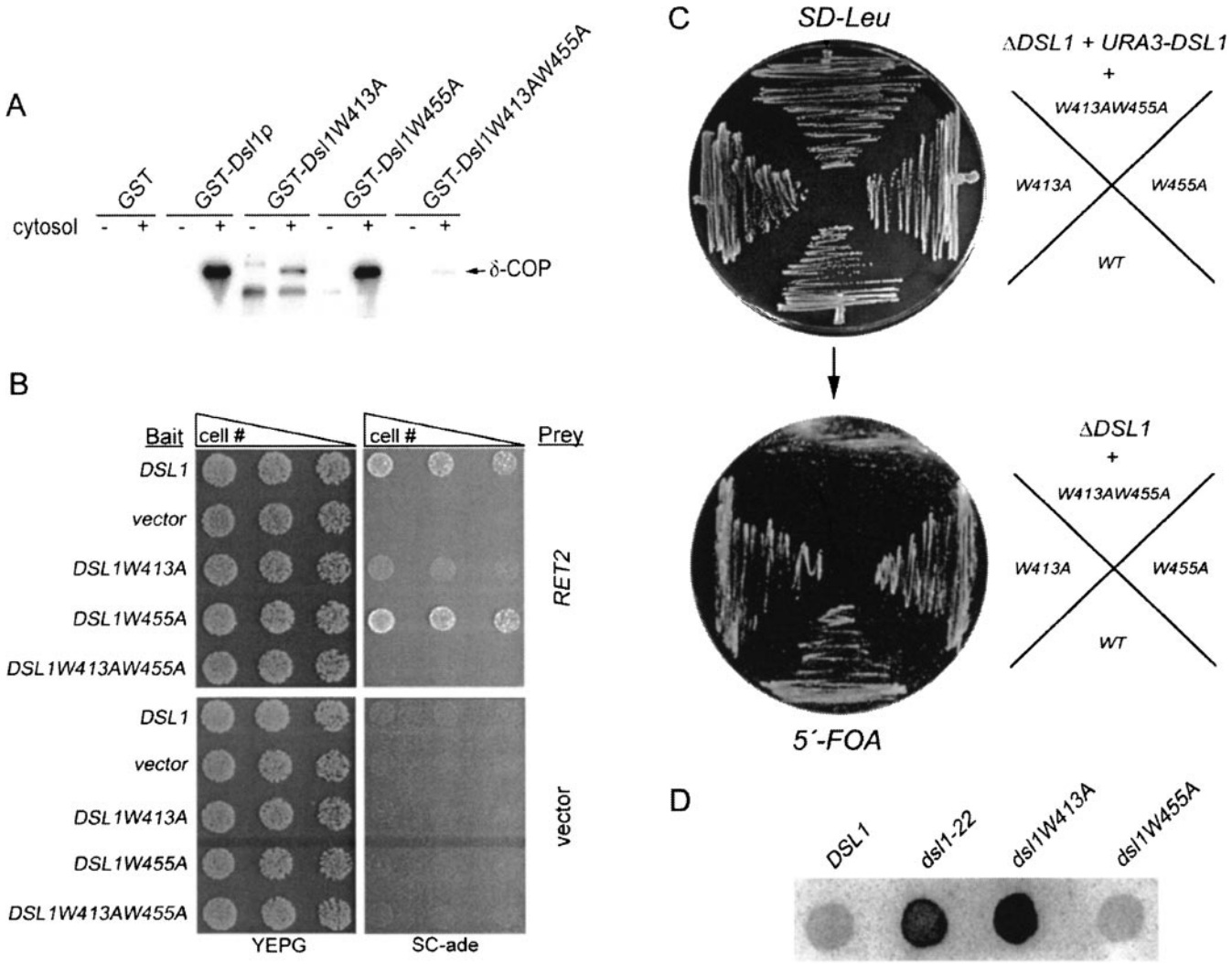


FIG. 3. **Alanine substitutions of Trp-413 (A*X*W) and/or Trp-455 (A*XXX*W) within Dsl1p (full-length) result in a decrease of δ-COP binding and growth defects.** *A*, GST-Dsl1p/δ-COP binding assay performed as described under "Experimental Procedures." δ-COP was visualized by immunoblot analysis. *B*, two-hybrid interaction analysis of δ-COP (*RET2*) with Dsl1p mutated in the δ-COP binding domain. The *S. cerevisiae* reporter strain PJ69–4A was co-transformed with plasmids encoding fusion proteins with the Gal4p-binding domain (*-BD*; bait) or the Gal4p-activation domain (*-AD*; prey) as indicated (33). Serial dilutions of cells were spotted onto rich media (YEPG) to assess cell viability and on synthetic complete medium lacking adenine (*SC-ade*) to detect two-hybrid interaction. Transformants containing either the empty bait (*upper panel*, *second row*) or prey plasmid (*lower panel*) served as negative control. *C*, plasmid shuffling assays proving that the *dsl1*$^{W413A/W455A}$ mutation is lethal. Δ*dsl1* cells expressing wild type *DSL1* from a *URA3* vector were transformed with *LEU2* plasmids (pRS315) carrying the indicated wild type/mutant versions of *DSL1*. Transformants were plated onto 5′-FOA plates selective for cells able to loose the *URA3* + *DSL1* carrying plasmid. The ability to grow on these plates indicates that a mutant allele is functional. *D*, BiP/Kar2p secretion assay. Log phase Δ*dsl1* cells expressing either wild type *DSL1*, *dsl1–22*, *dsl1*^{W413A}, or *dsl1*^{W455A} mutant alleles from a low copy plasmid were spotted onto nitrocellulose placed on top of rich media plates. After growth at 33 °C for 18 h, the nitrocellulose was washed thoroughly and secreted BiP/Kar2p was detected by affinity-purified α-HDEL antibody as described under "Experimental Procedures."

fused to the Gal4p-DNA binding domain served as bait. In this assay the formation of a complex between the GAL4 activation and GAL4 DNA binding domain confers prototrophy for adenine. Fig. 3*B* shows that the expression of the W455A substitution (A*XXX*W) resulted in growth similar to wild type, whereas the W413A (A*X*W) substitution led to a weaker interaction and therefore much slower growth. Consistent with the data from pull-down experiments, substitution of both tryptophan residues (W413A + W455A = A*X*W . . . A*XXX*W) reduced the growth rate to that of the vector control.

In Vivo Effects of Alanine Substitutions at Trp-413 and Trp-455 in Dsl1p—We wanted to determine whether the strong effect of the Trp → Ala substitutions observed *in vitro* is mirrored by phenotypic changes *in vivo*. Therefore, we performed a plasmid shuffling experiment. The mutations described above were introduced into the *DSL1* sequence present on a low copy plasmid (*LEU2* marker). The plasmids obtained were transformed into Δ*dsl1* cells expressing a wild type *DSL1* from another vector carrying the *URA3* marker. Cells harboring the latter plasmid are Ura$^+$ and thus sensitive to the drug 5-FOA (39). After replica plating onto 5-FOA-containing plates, cells were analyzed for growth. Only those cells that were able to loose the *URA3* + *DSL1*-containing plasmid can survive. Both single mutants, *dsl1*^{W413A} and *dsl1*^{W455A} (A*X*W or A*XXX*W) showed growth on 5-FOA plates, suggesting that they can replace the wild type *DSL1* gene very efficiently (Fig. 3*C*). In contrast, the *dsl1*$^{W413A/W455A}$-expressing cells (A*X*W . . . A*XXX*W) were not able to form colonies on these plates. The apparent lack of function of the double mutation was confirmed independently; Δ*dsl1* spores (*dsl1*::*kanMX*) expressing this mutant allele from a centromeric vector could not form colonies. The same plasmid was also unable to suppress the Ts$^-$-phenotype of *dsl1–22* mutant cells (data not shown) (32).

The single Trp → Ala substitutions (W413A or W455A =

A*X*W or A*XXX*W) did not lead to visible growth defects in a temperature range from 14 to 37 °C (data not shown). We examined possible additive effects by combining the single mutations in *DSL1* with the *ret2–1* (δ-COP) mutation. Double mutants *dsl1*^{W413A}*ret2–1* and *dsl1*^{W455A}*ret2–1* grew very slowly at 25 °C and were unable to form colonies at 33 °C, a permissive temperature for the single mutants (data not shown). No synthetic interaction was observed when we combined the same *dsl1* mutations with a mutation bearing a defect in the γ-COP subunit of coatomer, *sec21–1* (40). This indicates that the synthetic growth defect observed with *dsl1*^{W413A}*ret2–1* and *dsl1*^{W455A}*ret2–1* mutants is specific. In contrast, the *dsl1–22* mutation showed synthetic interaction with both the *ret2–1* and the *sec21–1* mutation (32). Together these results showed that the single *dsl1*^{W413A} and *dsl1*^{W455A} mutants are not fully functional.

A hallmark of mutants affected in the Golgi-ER retrograde traffic mutants is the secretion of the soluble ER protein BiP/Kar2p (41). This is also true for *dsl1–22*, *dsl1–4*, and *dsl1–7* mutants (32, 33). We examined BiP/Kar2p secretion by wild type cells (*DSL1*), as well as *dsl1–22*, *dsl1*^{W413A}, and *dsl1*^{W455A} mutant cells. Cells were grown at 33 °C on nitrocellulose filters that were placed on top of rich media plates for 18 h. Subsequent immunodetection revealed that the extent of BiP/Kar2p of *dsl1*^{W413A} (A*X*W) cells was comparable with that of *dsl1–22* cells (Fig. 3*D*; Ref. 32). The *dsl1*^{W455A} (A*XXX*W) mutation did not increase BiP/Kar2p secretion above wild type level. This finding suggests that the decrease in δ-COP binding of *dsl1* mutants observed *in vitro* correlates well with the *in vivo* effects on growth and retrograde transport from Golgi to ER.

Mapping of the Dsl1p-binding Site within δ-COP—Reilly *et al.* (33) have isolated fragments of the δ-COP-encoding *RET2* gene in a yeast two-hybrid screen for Dsl1p interacting proteins. The results of this screen indicated that the central part of the protein (residues 156–398 of 546) contains the Dsl1p binding site. We could confirm and extend this observation by using purified proteins. GST-Dsl1p was produced in *E. coli* and then immobilized on glutathione-Sepharose beads for binding experiments. His_6-tagged versions of δ-COP produced in *E. coli* were applied to the GST-Dsl1p-carrying beads. A truncation of δ-COP, which removed the last 146 residues, did not affect the binding *in vitro* (Fig. 4*A*, *lane 6*). However, an N-terminal deletion extending to residue 280 reduced the interaction to background levels (Fig. 4*A*, *lanes 3* and *7*). *Lane 11* in Fig. 4*A* shows that a shorter N-terminal truncation up to residues 189 has no effect on the binding. Together, this suggested that the central part of δ-COP spanning ~100 residues around residue 240 might be required for the binding to Dsl1p. This region represents a non-conserved linker region within δ-COP, which connects the well conserved N- and C-terminal domains.

The Dsl1p binding sites in δ-COP mapped by us is upstream of the δ-COP fragment used by Cosson *et al.* (7) to identify the δL motif (W*XXX*W). In contrast to the acidic-tryptophan motifs in Dsl1p, the δL and δL-like motifs used by these authors were always located close to the C terminus of the coatomer-interacting proteins. Accordingly, we observed strong interaction of the His-tagged C-terminal δ-COP fragment (residues 281 to 576) with δL-like motif of Dsl1p (GST-L1; Fig. 2*A*; data not shown). As expected, the Trp → Ala substitution within the δL motif at position 455 abolished the binding of this peptide to the δ-COP fragment. This indicates that the δL-like motifs can interact with the C-terminal part of δ-COP as long as this motif is C-terminally exposed.

To further delineate the Dsl1p binding domain in δ-COP, we performed an overlay assay using an array of peptides covering the region of amino acids 191–302 of δ-COP (a total of 32 peptides, overlapping in sequence by 11 amino acids; Jerini, AG, Berlin, Germany). The results of this assay indicated that Dsl1p-interacting peptides locate to a segment between residues 242 to 272 of δ-COP (data not shown). This region is part of a unique domain consisting mainly of small amino acids (Gly, Pro, Ala, Ser, Thr) and six basic residues (no Asx, Glx, Tyr, Phe, Trp, Cys). We could confirm the results of the overlay assay by using peptides coupled to agarose. These peptides could bind GST-Dsl1p, and they could recruit GST-L4 containing the W*X*W motif very efficiently from *E. coli* lysates. Fig. 4 (*B* and *C*)

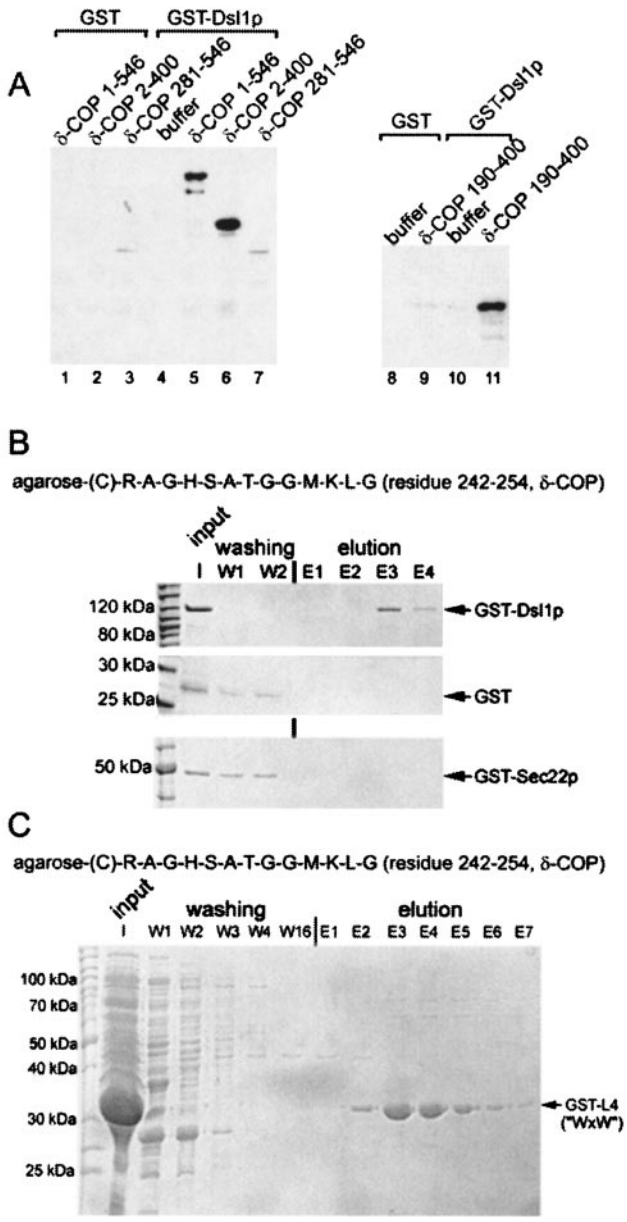


FIG. 4. **δ-COP interacts with Dsl1p via a central region of low structural complexity.** *A*, GST/GST-Dsl1p was immobilized on glutathione-Sepharose 4B and incubated with buffer, full-length recombinant His_6-tagged δ-COP (aa 1–546), and the following fragments: aa 2–400, 281–546, and 190–400. Samples were washed thoroughly (for details see "Experimental Procedures") and analyzed for bound δ-COP and δ-COP fragments by immunoblot analysis. *B* and *C*, the δ-COP-derived peptide R^{242}-A-G-H-S-A-T-G-G-M-K-L-G^{254} was immobilized on agarose and incubated with purified GST-Dsl1p, GST-Sec22p (*B*), or crude extract of GST-L4-expressing *E. coli* cells (*C*; see Fig. 2*A*) at room temperature. After washing thoroughly, bound proteins were eluted. Fractions of washing (*W*) and elution (*E*) steps were collected and analyzed by SDS-PAGE and Coomassie staining. GST, GST-containing chimeric proteins, and molecular size markers are indicated.

shows two examples of such binding experiments. To test the significance of these results, we introduced deletions into the δ-COP encoding *RET2* gene in yeast (see "Experimental Procedures"). Surprisingly, none of the deletions introduced into the region between residues 233 and 280 of δ-COP had an effect on the viability of cells. They also did not abolish the ability to bind to GST-Dsl1p (see Fig. 5*A* for some examples). This raises the possibility that the mutated δ-COP versions produced in yeast retain the ability to bind to GST-Dsl1p via an interaction with an unidentified Dsl1p-binding protein. To test this assumption, the mutated *RET2* genes were expressed from multicopy vectors. We reasoned that the binding efficiency of a mutant protein deficient in direct binding to Dsl1p could not be increased by overproduction. Only one of the mutant proteins behaved in this way (Fig. 5*D*). The overproduction of two of the deletion mutants (Δ13 and Δ27) led to an increase in the amount of protein bound to GST-Dsl1p similar to that of the wild type protein. In contrast, an equally low amount of Δ48 + 3HA mutant protein bound to GST-Dsl1p regardless of the expression level. In this mutant residues 233–280 had been replaced by three HA tags. The lower amount of this δ-COP-Δ48 + 3HA mutant protein was not the result of a reduced stability because the protein could be efficiently precipitated from the supernatant of a pull-down experiment (Fig. 5*C*). These results suggested that Δ13 and Δ27 deletion mutants have retained the ability to interact directly with GST-Dsl1p. In contrast, the binding of the Δ48 + 3HA mutant protein to GST-Dsl1p is indirect. This was confirmed later by showing that the Δ48 + 3HA-mutant protein can interact only with the W*X*W-containing GST-L4 fusion protein and not with GST-L1 (W*XXX*W). As shown below (Fig. 7*C*), GST-L1 is specific for δ-COP, whereas GST-L4 also interacts with a second coatomer subunit, thus allowing indirect binding of δ-COP to Dsl1p as part of the coatomer complex.

Dsl1p Also Interacts with α-COP—The results of the experiment shown in Fig. 5 prompted us to investigate whether Dsl1p has additional binding sites in the coatomer complex. Extracts from yeast cells deficient in particular COPI subunits were applied to GST-Dsl1p immobilized on glutathione Sepharose. Fig. 6*A* shows the result of an experiment using extracts from *ret2–1* mutant cells (δ-COP-defect), *sec28*Δ (ϵ-COP-deficient) as well as *ret2–1/sec28*Δ double mutants. In extracts from *ret2–1* cells, very little δ-COP can be detected even when cells were broken very rapidly and the lysate was mixed immediately with Laemmli buffer (*lane 2* in Fig. 6*A*). As shown by Duden *et al.* (42), ϵ-COP-deficient cells are viable and α-COP levels decrease dramatically when these mutants were shifted to 37 °C for 4 h. We also created a *ret2–1*/sec28Δ double mutant, which is lethal under normal growth conditions. However, the *ret2–1/sec28*Δ cells are viable when stabilized osmotically by the presence of 1 M sorbitol.[2] The pull-down experiment shown in Fig. 6*A* (compare *lane 2* in *upper panel* and *lane 5* in *lower panel*) demonstrates that most coatomer subunits can bind to GST-Dsl1p when the δ-COP level is so low that the protein cannot be detected by immunoblotting using either anti-δ-COP or anti-coatomer antibodies. This was consistent with the assumption that coatomer contains more binding sites for Dsl1p besides δ-COP. Little coatomer bound to GST-Dsl1p in the absence of ϵ-COP (Fig. 6*A*, *lane 6*). This residual binding activity does not depend on δ-COP because the high molecular weight coatomer subunits interacted with GST-Dsl1p when extracts of ϵ-COP mutants were used that also carried the *ret2–1* defect (Fig. 6*A*, *lane 8*). The observation that δ-COP can bind independently to Dsl1p is also confirmed by these experiments. δ-COP is the only subunit recruited from an extract of ϵ-COP-deficient cells grown for 4 h at 37 °C (Fig. 6*A*, *lane 7*). The cell extracts used for the pull-down samples shown in these *last two lanes* of Fig. 6*A* contained very little and mainly degraded coatomer (data not shown). However, the strong bind-

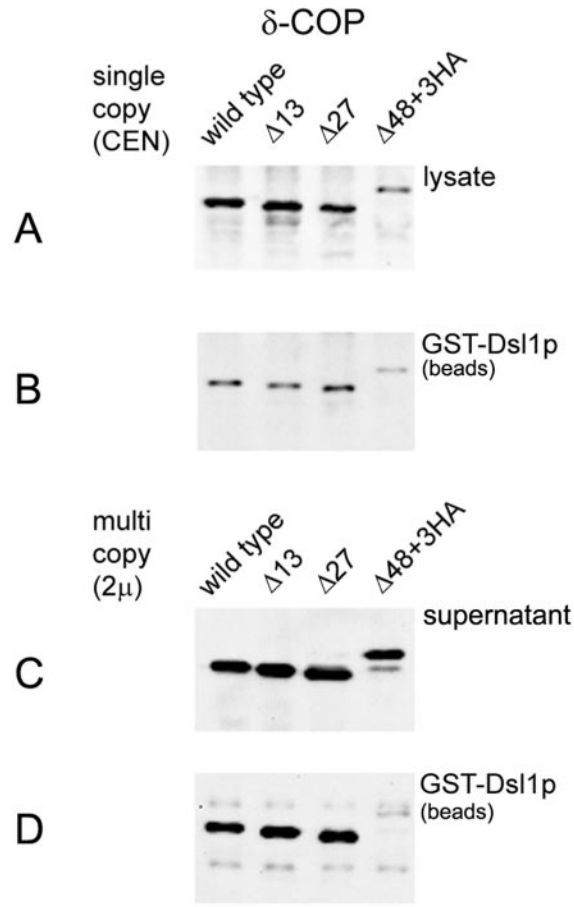


FIG. 5. **δ-COP may bind indirectly to Dsl1p.** GST-Dsl1p was immobilized on glutathione-Sepharose and used for pull-down experiments with lysates from yeast. After SDS-PAGE and blotting onto nitrocellulose, anti-δ-COP antibodies were applied to visualize δ-COP of aliquots of cell extracts or supernatants (*A* and *C*) and washed Sepharose beads (*B* and *D*). *A*, immunoblot analysis of cell extracts from wild type cells and *ret2*Δ (δ-COP) cells expressing *RET2* mutants carrying deletions in the putative Dsl1p-binding region, Δ13, Δ27, and Δ48 + 3HA. Δ13 lacks residues 242–254, the stretch of residues, which gave the strongest and most specific signal in the overlay assay with GST-Dsl1p. The Δ27 mutant carried an additional deletion of 14 residues (262–275). In mutant Δ48 + 3HA, residues 233–280 had been replaced by three HA tags. The amount of cell extract analyzed represents one tenth of the amount of extract loaded on GST-Dsl1p-carrying beads. The *RET2* versions were expressed from low copy number (CEN) vectors. *B*, proteins from extracts shown in *A*, which bound to GST-Dsl1p during the pull-down experiment. Half of the pellet fraction was loaded on the gel. *C*, immunoblot analysis of the aliquots corresponding to 1/10 of the supernatants, which were collected after the pull-down experiment. This was done to rule out the possibility that the mutant protein is unstable. Extracts applied to immobilized GST-Dsl1p were from strain BY4742 harboring multicopy vectors (2 μm) for overexpression of *RET2* and the deletion mutants listed in *A*. Transformants were grown in selective minimal medium. A small amount of wild type δ-COP encoded by chromosomal wild type gene is visible in the *last lane*. *D*, GST-Dsl1p pull-down experiment using extracts shown in *C* as described for *B* of this figure. Note that, in case of the Δ48 + 3HA mutation, a constant amount of mutant protein bound to GST-Dsl1p, which did not depend on the expression level. Note that δ-COP has much slower mobility during SDS-PAGE as predicted from the sequence (546 residues; see Fig. 2*B*). δ-COP often runs very close to Dsl1p and Tip20p-*myc* (both more than 80 kDa; see Footnote 2). Some of the changes we made, for instance the Δ48 + 3HA mutation, aggravated this unusual behavior.

[2] H. D. Schmitt, unpublished observation.

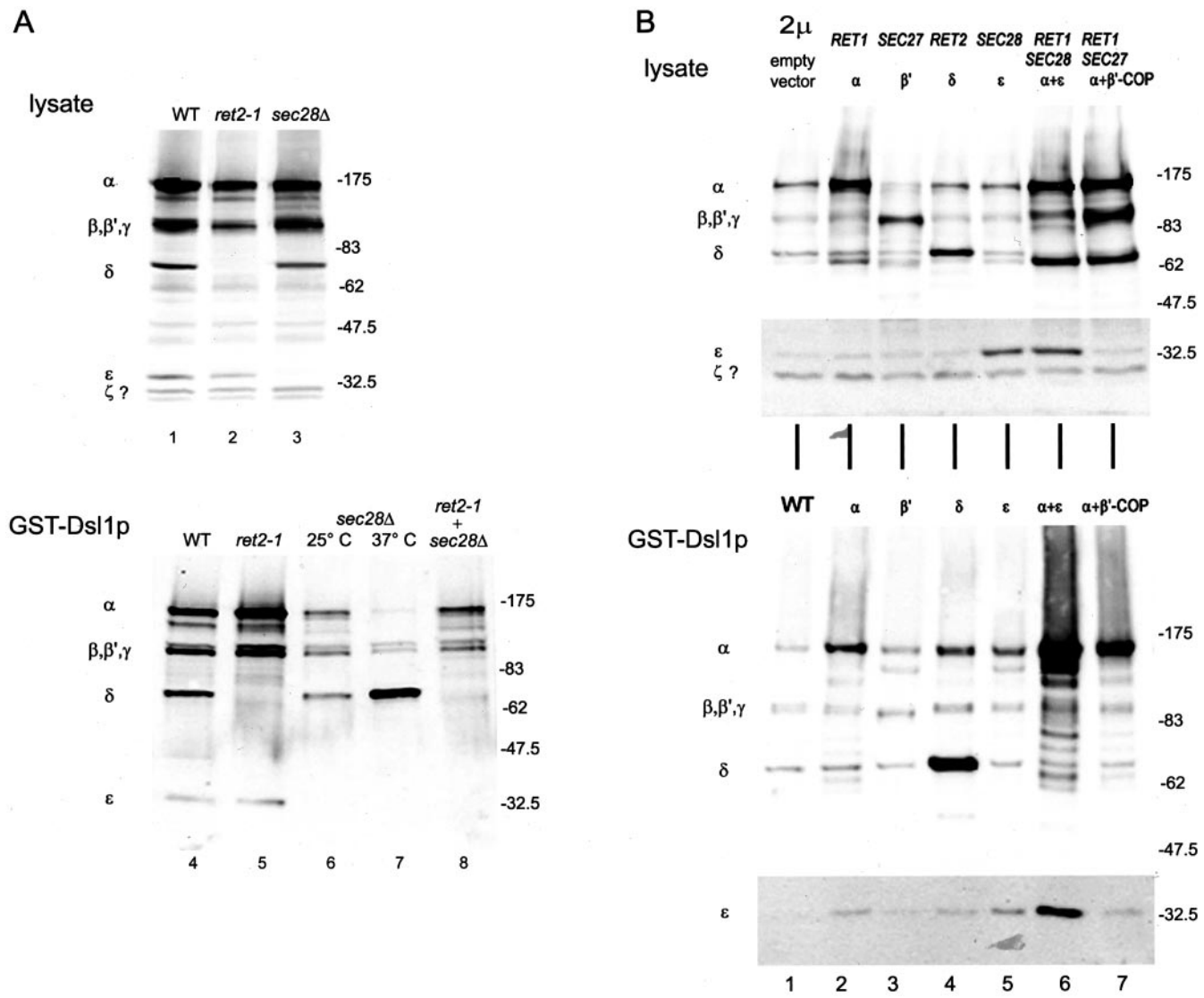


FIG. 6. **Dsl1p interacts with α-COP.** Anti-coatomer antibodies (38) were used for immunoblot experiments with yeast extracts and samples from pull-down experiments using immobilized GST-Dsl1p. *A*, *lanes 1–3*, immunoblot analysis of coatomer from wild type cells (BY4742), a *ret2–1* mutant (strain R2P4–5C; δ-COP-defect), and a *sec28*Δ mutant (strain Y11469; ϵ-COP-defect) grown at 25 °C; *lanes 4–9*, GST-Dsl1p was immobilized on glutathione-Sepharose and used for pull-down experiments with lysates from yeast. The strains used for this experiment were spores of tetrad derived from a cross of *ret2–1* and *sec28*Δ cells (see above). This cross was performed to obtain the *ret2–1*/*sec28*Δ double mutants. Double mutant cells from several tetrads had to be collected and grown in 1 M sorbitol medium to get enough starting material. *sec28*Δ mutants were either grown at 25 °C or shifted to 37 °C for 4 h to deplete cells from α-COP as described by Duden *et al.* (42). Yeast extract equivalent to 200 μg of protein were incubated with 7.5 μg of GST-Dsl1p immobilized on glutathione-Sepharose at room temperature. After a 1.5-h incubation, samples were washed five times with buffer B88. Bound proteins were eluted with SDS buffer and analyzed by SDS-PAGE and immunoblotting. *B*, *upper panel,* immunoblot analysis of extracts from BY4742 cells overexpressing coatomer subunits. Cells harboring either the empty vector pRS323, single 2-μm vectors or combinations of two 2 μm vectors carrying COPI genes as indicated were grown in selective minimal medium. The plasmids are listed in Table II. *Lower panel*, GST-Dsl1p pull-down experiment using extracts shown in the *upper panel* as described for *A*. The intensity of bands in the *lower parts* of each *panel* was increased using imaging software to improve the visibility of ϵ-COP and ζ-COP.

ing of δ-COP or the high molecular weight subunits contained in these extracts showed that single subunits or partial complexes interact more efficiently with Dsl1p than the whole complex.

Several coatomer mutants like *cop1–1* (= *ret1–1*; α-COP), *sec21–1* (γ-COP), *sec27–1* (β′-COP), and *ret3–1* (ζ-COP) did not show a change in the pattern of subunits, which bind to GST-Dsl1p (data not shown). Experiments using γ-COP- and β′-COP-specific antibodies were performed to confirm that these subunits bind to GST-Dsl1p as effectively as δ-COP. The extracts used for this experiment were from wild type cells (data not shown). No protein with the size of ζ-COP bound to GST-Dsl1p. Either ζ-COP may be lost from the complex during the washing procedure or Dsl1p and ζ-COP may compete for similar binding sites in coatomer. For reasons mentioned below, it will be necessary to use ζ-COP produced in *E. coli* to address this question. Another coat component that was not found attached to the GST-Dsl1p-carrying beads were the ARF proteins.[2]

The efficient binding of coatomer subunits other than δ-COP to Dsl1p may require the combination of two or more subunits. The results presented in Fig. 6A, for instance, indicated that ϵ-COP may act as a factor that facilitates the binding of other subunits without being directly involved in binding. To obtain partial coatomer complexes, we tried to overproduce combinations of coatomer subunits. An increase in the amount of γ-COP and ζ-COP could not be achieved. Even the co-transformation of multicopy (2 μm) plasmids carrying either *SEC21* (γ-COP) or *RET3* (ζ-COP) into protease-deficient strains had no effect. In contrast, the presence of the *COP1* (*RET1*) gene on 2-μm vector

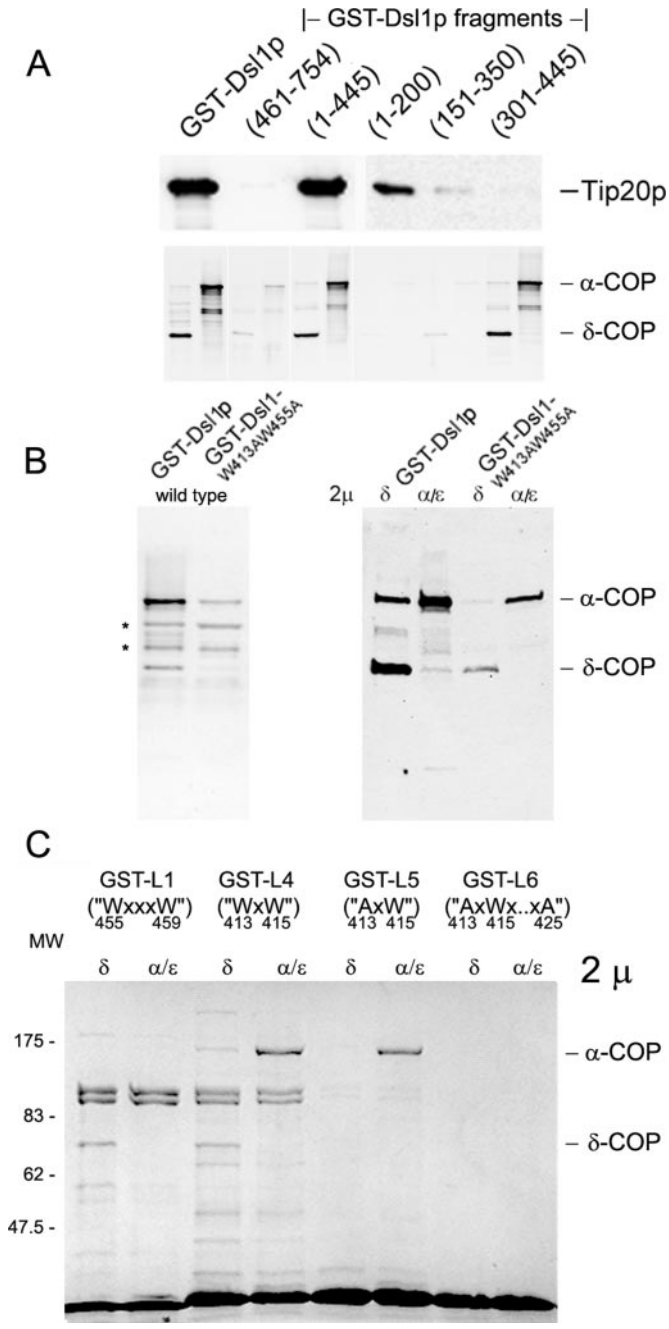


FIG. 7. **Mapping of the binding site for Tip20p, δ-COP, and α-COP in Dsl1p.** *A*, GST fusions of either full-length Dsl1p or truncated versions of Dsl1p purified from *E. coli* were immobilized on glutathione-Sepharose 4B. After incubation with extract from detergent-lysed yeast cells at 4 °C, beads were washed thoroughly and the proteins retained were analyzed by SDS-PAGE and immunoblot analysis. *A, upper panel*, extracts of wild type cells overexpressing Tip20-*myc* (28) were applied to GST fusion proteins, which had been immobilized on glutathione-Sepharose; *lower panel*, extracts from *sec28*Δ grown at 37 °C for 4 h and *ret2–1* cells grown at room temperature were applied to the same GST fusion constructs as above. Proteins bound to beads were analyzed using polyclonal antibodies against the c-*myc* epitope (Santa Cruz) to visualize Tip20p-*myc* (*upper panel*) or anti-coatomer antibodies (*lower panel*). The bands shown in the *lower panel* were all from the same experiment and arranged in a way that they can be compared directly to the Tip20-*myc* pull-down. *B*, comparison of α-COP and δ-COP binding to GST-Dsl1p and GST-Dsl1p carrying two tryptophan-alanine substitutions in the central acidic domain. The fusion proteins GST-Dsl1p and GST-Dsl1p$^{W413A/W455A}$ were immobilized on glutathione Sepharose and used to pull down proteins from wild type extracts or extracts from δ-COP- or α/ε-COP-overproducing cells as described above. Anti-coatomer antibodies were used to visualize the coatomer subunits, which had bound to the beads. The *asterisks* mark GST-Dsl1p and a fragment derived from it, which cross-reacted with anti-coatomer antibodies. These antibodies were removed from the serum used for the other experiments as described under "Experimental Procedures." Here they may serve as an internal control. *C*, a Coomassie-stained polyacrylamide gel showing proteins, which bound to Dsl1p-derived peptides from the central acidic domain fused to GST. Most of the fusion constructs were listed in Fig. 2. GST-L6 carries an additional tryptophan-alanine replacement at the position that corresponds to Trp-425 in the full-length protein. The strong bands between δ-COP and α-COP represent subunits of the phosphofructokinase as shown by mass spectroscopy, whereas p55 visible in Fig. 2 is the aldehyde dehydrogenase. The varying binding efficiencies depended on whether the δ-COP- and α/ε-COP-overproducing cells had grown exponentially or were at stationary phase.

led to a considerable increase in the amount of α-COP in extracts (Fig. 6*B*, *upper pan*el). The effects of this overproduction on the efficiency of α-COP binding to GST-Dsl1p were quite variable. On the average only a 3-fold increase in binding efficiency was observed, whereas in the experiment shown in Fig. 6*B* (*lower panel*), the overproduction of α-COP alone resulted in an 8-fold increase in binding. Overexpression of *SEC27* or *SEC28* resulted in an increase in β′-COP and ε-COP in the cells, but this did not change the amount of these subunits bound to GST-Dsl1p (Fig. 6*B*). The simultaneous increase in the amount of α-COP and ε-COP in the extract of double transformants dramatically improved the binding of both subunits to GST-Dsl1p. Quantification of the bands showed that the increase in α-COP bound to GST-Dsl1p was more than 60-fold. The increase in ε-COP binding was ~30-fold. Surprisingly, a surplus of β′-COP also improved the binding of α-COP, although the amount of β′-COP bound to GST-Dsl1p was not increased at all. The most likely interpretation of the results shown in Fig. 6 is that α-COP can interact with GST-Dsl1p directly and that binding is improved when α-COP can adopt a suitable conformation. The finding that ε-COP and β′-COP can induce this conformation is consistent with the previously made observation that α-COP, β′-COP, and ε-COP contact each other and form the so-called B subcomplex of the COPI coat (6, 43–45).

Mapping of the Binding Sites for α-COP and Tip20p in Dsl1p—Next, we wanted to determine the binding site for α-COP and Tip20p in Dsl1p and whether these sites may overlap with the binding site for δ-COP as determined above. GST fusion proteins containing different fragments of Dsl1p were incubated with extracts of yeast cells expressing a c-*myc*-tagged version of Tip20p at high levels (28) or with extracts from mutant cells. As shown above, extracts from *sec28*Δ mutant cells grown at 37 °C (lacking ε-COP and α-COP; Ref. 42) or *ret2–1* (δ-COP-deficient) mutant cells are suitable to analyze separate recruitment of α-COP and δ-COP by immobilized GST-Dsl1p. The *left half* of Fig. 7*A* shows that the binding sites for Tip20-myc as well as the two coatomer subunits are within the N-terminal 445 residues of Dsl1p. We then analyzed three overlapping segments of the N-terminal part (residues 1–200, 151–350, and 301–445). The *right half* of Fig. 7*A* shows that the binding sites for Tip20-myc are within the first 200 residues of Dsl1p, whereas both coatomer subunits interact with the fragment derived from the central part of Dsl1p.

The results presented in Fig. 7*A* suggested that α-COP and δ-COP may share a common binding site within Dsl1p. Therefore we tested whether the same mutations that disturb the binding of δ-COP to Dsl1p (see Fig. 3*A*) also disturb the interaction of α-COP with Dsl1p. Pull-down experiments were performed to compare the binding of coatomer from wild type cells as well as from δ-COP- or α/ε-COP-overproducing cells to GST-Dsl1p or GST-Dsl1p carrying the W415A/W455A double mutation. The results of these assays indicated that the double mutation affects the binding of α-COP as well, but not as

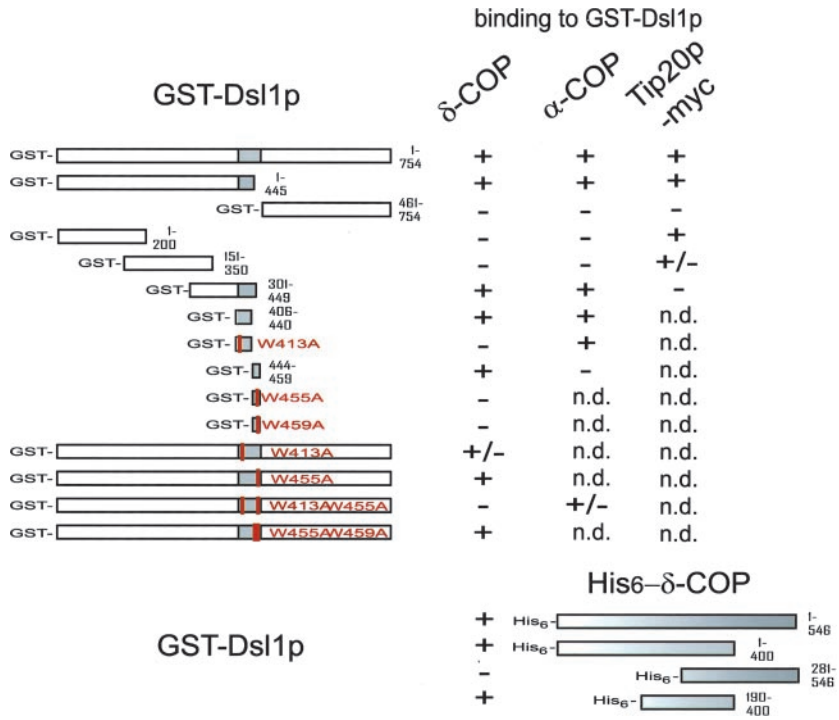


FIG. 8. **Overview of the deletion constructs used for mapping of interaction sites within Dsl1p for δ-COP, α-COP, and Tip20p.** The position of point mutations and the results of binding assays obtained in this study are indicated. *n.d.*, not determined.

strongly as that of δ-COP (Fig. 7*B*). To analyze the specificity for different tryptophan residues within the acidic-tryptophan region of Dsl1p in more detail, we employed the GST-peptide constructs used above (Fig. 2). Indeed, α-COP and δ-COP had different preferences for these peptides (see Fig. 7*C*). In most experiments only very little α-COP from α/ϵ-COP-overexpressing cells interacted with GST-L1 (W*XXX*W), the construct that resembles the δL motif (Ref. 7; see Fig. 2). Immunoblotting was necessary to visualize α-COP bound to this fragment (data not shown). In contrast, GST-L4 (W*X*W) could recruit enough α-COP from the cell lysate to be seen by Coomassie staining. Surprisingly, the α-COP could still bind to the A*X*W mutant (GST-L5). An additional mutation replacing the third tryptophan residue by alanine (position 425 in full-length Dsl1p) was necessary to prevent binding of α-COP. Recruitment of α-COP to GST-L4 is also most efficient if the extracts are made from cells overproducing both α-COP and ϵ-COP (data not shown).

Fig. 8 represents a summary of all the results obtained by *in vitro* binding experiments. An important result of the mapping experiments is the finding that the Tip20p binding site (residues 1–200) does not overlap with the coatomer-binding site within Dsl1p (residues 410–460). In contrast, δ-COP and α-COP make contact with overlapping sequence motifs within the central acidic domain of Dsl1p.

DISCUSSION

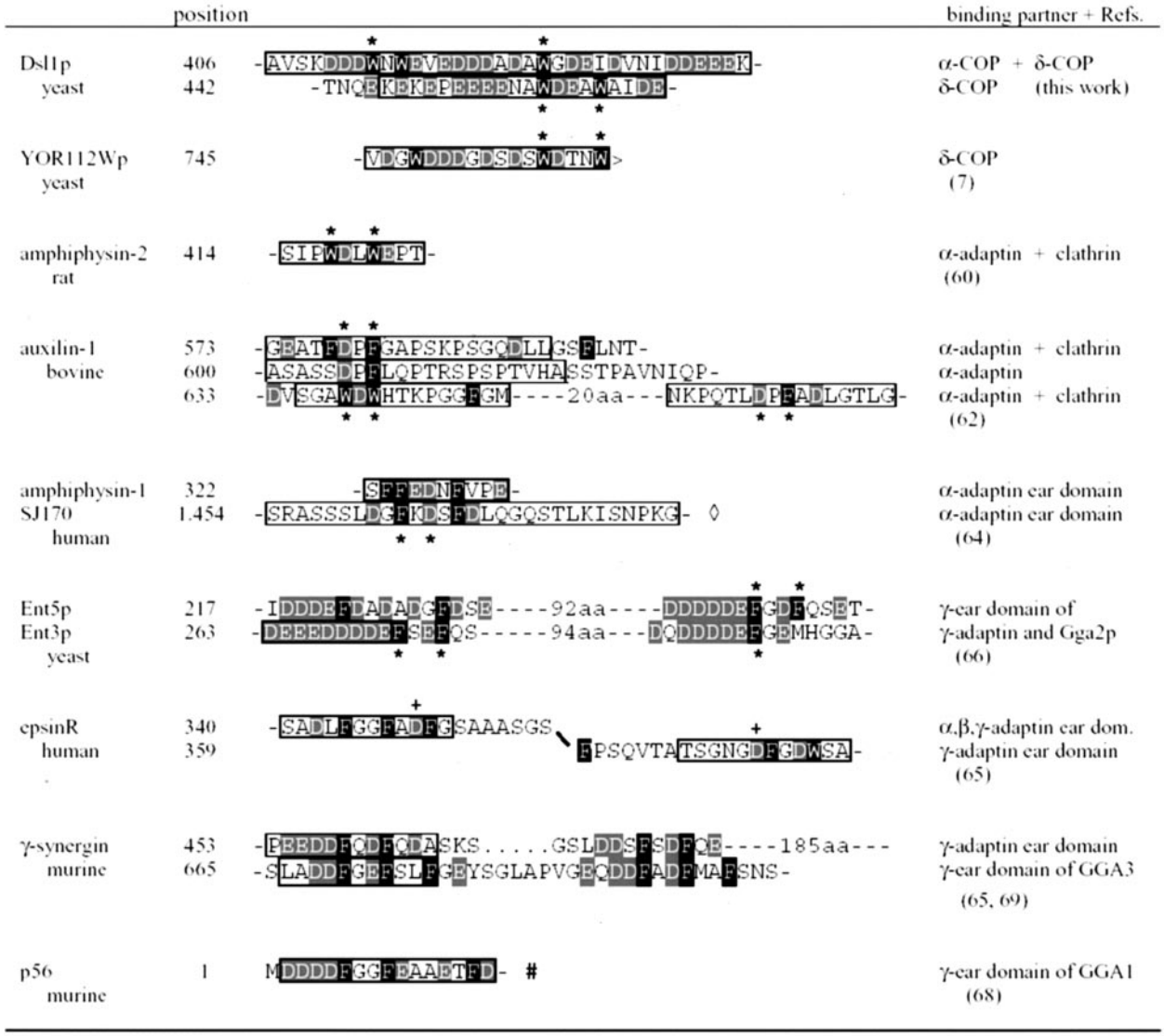
In this study we have characterized the interaction between three different factors involved in retrograde transport from Golgi to ER in yeast: the coatomer complex, as well as two peripheral ER proteins, Dsl1p and Tip20p. We were able to confirm and extend previous observations using recombinant proteins. The δ-COP/Dsl1p interaction could be reconstituted *in vitro* proving results obtained by Reilly *et al.* (33), who had isolated the δ-COP encoding *RET2* gene in a yeast two-hybrid screen with *DSL1* as bait. Two-hybrid data as well as immunoprecipitation experiments had shown that Dsl1p interacts with Tip20p, a component of the membrane fusion machinery at the ER (33, 34). Our results indicate that the N terminus of Dsl1p is involved in this interaction.

Attempts to map the binding sites within Dsl1p and δ-COP indicated that both proteins interact through regions of low complexity present in the central parts of their sequence. Two di-aromatic motifs in the central aspartate- and glutamate-rich regions of Dsl1p are important for δ-COP binding and viability. The second of these two motifs (W*XXX*W) very much resembles the δL motif found by Cosson *et al.* (7). However, mutational analysis showed that a di-aromatic motif (W*X*W) located upstream of the δL-like motif in Dsl1p is more important for the binding of δ-COP. We found that this motif can also mediate the interaction with α-COP. The results shown in Fig. 7*C* indicated that the interaction with α-COP also involves a tryptophan residue that lies between the W*X*W and W*XXX*W motifs at position 425 of Dsl1p. Data base searches showed that many fungi encode a Dsl1p homolog with a central acidic domain (see supplemental material, available in the on-line version of this article). The acidic part of the Dsl1 proteins usually contains five or six tryptophan residues. Only the Dsl1p homolog from *Candida albicans* has three tryptophan residues in this low complexity region. The equivalent region from Dsl1 homologs from basidiomycetous fungi can have three WGF motifs in addition to one W*XXX*W motif.

Dsl1 proteins from filamentous fungi are homologs of the ZW10 proteins from higher eukaryotes (Ref. 46; see supplemental data). The fungal Dsl1 proteins and the ZW10 proteins show the highest degree of sequence conservation at the C terminus. This part of the ZW10 proteins is known to bind a subunit of the dynein recruiting dynactin complex (47). It is tempting to speculate that this domain in Dsl1p from yeast may also function in the recruitment of cytoskeletal elements. This region has an important function because the previously characterized *dsl1–22* mutation is a nonsense mutation that removes the last 30 residues from Dsl1p (32). The alignments of fungal Dsl1 proteins and ZW10 from higher eukaryotes showed that the low complexity region mediating coatomer binding is unique to fungi.

The data base searches also revealed unexpected support for a functional link between Dsl1p and δ-COP. *Schizosaccharomyces pombe*, like *S. cerevisiae* an ascomycetous fungus, for which the whole genome is known (48), may be unique among

TABLE III
Acidic di-aromatic motives involved in the interaction with coat proteins



DE acidic residues, WF tryptophan or phenylalanine, ☐ peptides binding coat proteins, * analyzed by alanine substitution, + analyzed by arginine substitution, # peptide used in competition experiments, ◊ a surplus of this peptide prevents the binding of AP180 to the α-adaptin ear domain. AP180 contains three FxDxF motives (64).

fungi, because no *DSL1*-like gene in the genome of this yeast can be identified. It is also exceptional because it encodes a truncated version of δ-COP lacking the putative C-terminal cargo-binding domain. The absence of this domain may alleviate the need for a Dsl1p-like protein.

We could not precisely define the actual binding site for Dsl1p in δ-COP. Peptides derived from a central low complexity region in the central part of δ-COP can mimic the δ-COP-Dsl1p interaction. However, the absence of the corresponding segments from δ-COP produced in yeast did not affect the binding to Dsl1p. To disturb the direct interaction, it was necessary to insert bulky HA tags with several tyrosine and acidic residues into the central domain consisting primarily of small and basic residues. The removal of the HA tags restored normal binding characteristics even though 33 residues were still deleted from the protein.[3] This suggests that elements of the secondary structure of native δ-COP are most important for the interaction. The binding observed with peptides may represent intermediate steps in the assembly of the complex. It is still possible that the crucial binding site used *in vivo* resides in the C-terminal part of δ-COP (7). As we observed, this site is accessible for proteins with the W*xxx*W motif exposed at the C terminus. Yeast cells may contain a factor that can make this site accessible to proteins, which carry these motifs in the central part of their sequence. Similarly, Dsl1p requires additional subunits to bind to α-COP.

Dsl1p Interacts with Parts of the Coatomer Complex That May Belong to Different Layers of the Coat Complex—The most important finding of this study is the fact that Dsl1p uses overlapping binding motifs to interact with δ-COP as well as α-COP. Strong binding of α-COP to Dsl1p requires the simultaneous overexpression of at least one of its binding partners of the coatomer complex either ϵ-COP or β′-COP. The W413A/W455A mutation of Dsl1p may be lethal because it affects

[3] M. Essid and H. D. Schmitt, unpublished observation.

binding to both α-COP and δ-COP although to varying extents (Fig. 7*B*). In contrast, δ-COP mutants affected in Dsl1p binding may be viable because Dsl1p can still interact with α/ϵ-COP. To determine which interaction is more important, it will be necessary to identify the target site of Dsl1p in α-COP. As in case of δ-COP, the mapping of the Dsl1p-binding site in α-COP will require the use of recombinant α-COP and ϵ-COP.

The composition of coatomer subcomplexes has been determined by *in vitro* assembly and disassembly experiments (43, 49), as well as two- or three-hybrid analysis (44, 45, 50). According to the cargo that is recognized by the subcomplexes, they were named F and B subcomplex (6). Each constituent of the F subcomplex, β-, γ-, δ-, and ζ-COP, shares sequence similarities with subunits of the clathrin adaptor complexes (51–53). α-, β′-, and ϵ-COP constitute the B subcomplex, which is thought to be equivalent to clathrin itself. α- and β′-COP carry one copy of the clathrin heavy chain repeat (45, 54) and the N-terminal domains of α- and β′-COP can form a β-propeller structure like the clathrin heavy chain (1, 38, 55). By analogy, this suggests that Dsl1p can make contacts with components of both layers of the COPI coat. This is a feature shared by many accessory factors that assist the clathrin coat assembly during endocytosis (56, 57). The interaction with two layers in a vesicle coat may allow these proteins to induce rearrangements. In amphiphysin, synaptojanin170 (SJ170), epsin 1, HIP1, and auxilin, the binding sites for clathrin and the adaptor complex are arranged in tandem (58–62). Like Dsl1p these proteins rely on sequence motifs consisting mainly of hydrophobic and acidic residues for their interaction with the coat proteins (63, 64). Moreover, short peptides derived from amphiphysin and auxilin are able to mediate interaction with both clathrin and AP-2. The important elements in these peptides with dual specificity resemble the coatomer binding motifs from Dsl1p very much (P**W**DL**W** and **W**D**W**; Refs. 60 and 62; see Table III). Other adaptor binding motifs containing two aromatic residues were identified recently (**F***X*D*X***F**; **F***X*D**F**; Refs. 64–66). The **F***X*D**F** motif was identified in proteins from higher eukaryotes and yeast, epsinR, γ-synergin, p56, Ent3/5p (65–69; Table III). These proteins use the **F***X*D**F**-related motifs for interacting with AP-1, in particular the ear domain of γ-adaptin or GGA proteins. Most of these proteins contain two copies of this γ-ear binding motif. As in case of the coatomer-binding motif from Dsl1p, the **F***X*D**F** sequence is embedded in long stretches of aspartate and glutamate residues. Another recently identified binding partner of the γ-ear domain of the mammalian AP-1 complex is Snx9 (70). It carries a stretch of residues between the SH3 and the PX domain, D-D-D-D-**W**-D-E-D-**W**-D, which resembles the acidic-tryptophan motif from Dsl1p even better. We predict that this motif is responsible for the binding to the adaptor complex.

Dsl1p and the accessory proteins of the clathrin coat differ in one important respect. The target site for most accessory proteins are the C-terminal ear domains of the α- or γ-adaptin, whereas Dsl1p binds to δ-COP, the equivalent of the adaptor medium chain (μ1- or μ2-chain). The μ2-chain is known as cargo receptor for proteins carrying the **Y***XX***Φ** sorting motif (71) and hence δ-COP was supposed to function as a cargo receptor as well. In fact, Cosson *et al.* (7) presented evidence that the δL motif (**W***XXX***W**) can function as a retrieval signal. Therefore, one can speculate that sorting signals and the motifs involved in the binding of accessory factors to coat complexes may have evolved from the same ancestral coat binding motif. In contrast to the COPI coat, the large adaptor subunits may have developed the C-terminal ear domains as the specialized platforms for the interaction with other adaptors or accessory proteins. The Dsl1p/coatomer interaction may represent a rather ancient state in evolution of coat/adaptor complexes because Dsl1p still makes contact with the putative cargo binding subunit.

Another COPI/clathrin-adaptor resemblance concerns the binding strength of cargo-specific and the acidic-tryptophan motifs. A direct comparison shows that the peptides containing the cargo-specific **KK***XX* and **Y***XX***Φ** motifs recruit coat proteins much less effectively than the W*XXX*W-, W*X*W-, and P**W**DL**W**-including peptides (7, 60).[2]

Dsl1p as Part of a Putative Tethering Complex—Binding of Dsl1p to Tip20p was observed previously by co-immunoprecipitation and yeast two-hybrid analysis (33, 34). Tip20p is associated with the retrograde Golgi-ER SNARE complex (25, 28–30). Dsl1p contains a leucine zipper motif close to the N terminus (72) that may be able to form coiled-coil structures. N-terminal coiled-coil regions are characteristic for components of some tethering factors (21). It is likely that additional factors are involved in the Dsl1p-Tip20p interaction because Dsl1p is part of a large complex (33). Together these proteins may constitute a complex that initiates docking of Golgi-derived vesicles at the ER.

In Dsl1p the binding site for coatomer and Tip2p do not overlap, and Tip20-myc binds to GST-Dsl1p with equal efficiency regardless of whether large amounts of α-COP were bound to it or not.[2] This suggests that a simultaneous interaction of Dsl1p the peripheral ER protein Tip2p and the COPI complex is possible. It is not without precedent that a subunit of a tethering factor interacts with a coatomer subunit (73). Moreover, there is evidence that factors involved in vesicle formation or sorting are required for membrane fusion as well (74, 75). In particular, Vps41/Vam2p as a constituent of the AP3 coat is involved in vesicle formation at the Golgi (76). Vps41/Vam2p is also a subunit of the VPS-C/HOPS complex, a tethering complex involved in vacuole fusion (18). It will be important to determine whether Dsl1p acts as a direct link between a docking and a coat complex.

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ADDITIONS AND CORRECTIONS

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Dsl1p, an essential component of the Golgi-endoplasmic reticulum retrieval system in yeast, uses the same sequence motif to interact with different subunits of the COPI vesicle coat.

Uwe Andag and Hans Dieter Schmitt

On page 51726, we misleadingly stated that the central acidic domain in Dsl1p is essential for viability. Evidence for this was presented in Fig. 3*C*. This figure shows that a mutant carrying two Trp-to-Ala replacements in this region did not support growth of a *dsl1* deletion mutant. However, we recently created a GAL-regulated TAP-tagged version of *DSL1* carrying five Trp-to-Ala substitutions in this region to use as a negative control in pulldown experiments. Surprisingly, this mutant complemented the *dsl1* knock-out. Even a single-copy untagged version of this allele could replace the wild-type gene. However, these cells grow poorly at all temperatures tested and show phenotypes similar to those of Dsl1p-depleted cells.

Sequencing showed that the plasmid used for the growth assay in our previous work contained a C-terminal truncation in addition to the Trp-to-Ala substitutions at positions 413 and 455. A mutant clone with an intact C terminus supported growth of a *dsl1* deletion mutant.

Our recent data are still consistent with the notion that the outer tryptophan motifs in the acidic domain of Dsl1p (Trp^{413}/Trp^{415} and Trp^{455}/Trp^{459}) mediate binding to δ-COP, whereas the central tryptophan residue at position 425 is involved in the interaction with α-COP. Obviously, all motifs must be destroyed to affect the growth of cells.

We sincerely apologize for any confusion that this may have caused.

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Angiotensin II type 2 receptor blockade increases bone mass.

Yayoi Izu, Fumitaka Mizoguchi, Aya Kawamata, Tadayoshi Hayata, Testuya Nakamoto, Kazuhisa Nakashima, Tadashi Inagami, Yoichi Ezura, and Masaki Noda

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The ATPase activity of BfpD is greatly enhanced by zinc and allosteric interactions with other Bfp proteins.

Lynette J. Crowther, Atsushi Yamagata, Lisa Craig, John A. Tainer, and Michael S. Donnenberg

This article has been withdrawn at the request of the authors.

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Determination of *in vivo* dissociation constant, K_D, of Cdc42-effector complexes in live mammalian cells using single wavelength fluorescence cross-correlation spectroscopy.

Thankiah Sudhaharan, Ping Liu, Yong Hwee Foo, Wenyu Bu, Kim Buay Lim, Thorsten Wohland, and Sohail Ahmed

On page 13603, the following sentence should be added to the legend of Fig. 1: The SH3 in the N-WASP schematic indicates a polyproline sequence that binds SH3 domains.

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Autotaxin/lysopholipase D and lysophosphatidic acid regulate murine hemostasis and thrombosis.

Zehra Pamuklar, Lorenzo Federico, Shuying Liu, Makiko Umezu-Goto, Anping Dong, Manikandan Panchatcharam, Zachary Fulkerson, Evgeny Berdyshev, Viswanathan Natarajan, Xianjun Fang, Laurens A. van Meeteren, Wouter H. Moolenaar, Gordon B. Mills, Andrew J. Morris, and Susan S. Smyth

Dr. Fulkerson's name was misspelled. The correct spelling is shown above.

Membrane Transport, Structure, Function, and Biogenesis:

Dsl1p, an Essential Component of the Golgi-Endoplasmic Reticulum Retrieval System in Yeast, Uses the Same Sequence Motif to Interact with Different Subunits of the COPI Vesicle Coat

Uwe Andag and Hans Dieter Schmitt

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