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

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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) 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 His6-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-agar 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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| Strain       | Genotype | Source          |
|--------------|----------|-----------------|
| MSUC-3B      | MATa, ade2, ura3, leu2, his3 | This laboratory |
| PJ-694A      | MATa, ura3-52, leu2-3,112, trp1-901, his3-200, ga14Δ, gal803, LYS2::GAL1-HIS3, GAL2::ADE2, met2::GAT1-lacZ | P. James |
| S2I9-2A      | MATa, leu2-2, his3, pep4::HIS3, sec21Δ-1 | This laboratory |
| S2I48-5C     | MATa, leu2, ura3, his3, lys2, trp1, his3, ret2-1 | This laboratory |
| Y21186       | MAT0ura, ura33000ura3Δ0, leu23000leu2Δ0, his3Δ1his3Δ1, lys2Δ0/LYS2, MET15/met15Δ0 | EUROSCARF |
| YUA1–9C      | MAT0, ade2, ura3, leu2, his3, lys2, del1–22 | This study |
| YUA12–1A     | MATa, ura3, leu2, his3, del1::KanMX, containing pUA89 (GFP-DSL1, URA3) | This study |
| BY4742       | MATa, ura3, leu2, his3, lys2 | EUROSCARF |
| Y11469       | MATa, sec28Δ (YIL076c::KanMX4), ura3, leu2, his3, lys2 | EUROSCARF |
| Y25865       | MAT0ura, ret2Δ (YFR055c::KanMX4/RET2, ura3::ura3, leu2::leu2, his3::his3, lys2::LYS2, MET15/met15Δ0) | EUROSCARF |
| R2P4–5C      | MATa, ret2–1, ura3, leu2, his3, trp1, lys2 | This study |

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 overexpressing 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 = WXXXW motif). The original ΔL motif is at the C terminus of the hypothetical protein YOR112w (D-D-...
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**Table II**

| 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 |
| pRS23-RET2 | RET2 (wild type or deletion mutants), HIS3, 2 μm | This laboratory |
| p22-RET2 | RET2 (wild type or deletion mutants), URA3, CEN | F. Letourneau |
| pRS23-RET1 | RET1 (COP1), HIS3, 2 μm | This laboratory |
| pRS23-RET1-S28 | RET1 (COP1), SEC28, HIS3, 2 μm | This laboratory |
| Ye24-SEC21 | SEC21, LEU2, 2 μm | R. Duden |
| pRS426-RET3 | RET3, URA3, 2 μm | B. A. Kraynack |
| Ye24-SEC27 | SEC27, URA3, 2 μm | R. Duden |
| Ye24-SEC28 | SEC28, URA3, 2 μm | R. Duden |
| pTM2 | TIP20-myC, CEN | M. Lewis |
| pUA73 | pRS35-DSL1, LEU2, CEN6/ARS4 | This laboratory |
| pUA81 | pRS25-DSL1, LEU2, 2 μm | This laboratory |
| pUA86 | pRS15-dsl1-22, LEU2, CEN6/ARS4 | This laboratory |
| pUA89 | pUG36-DSL1, URA3 | This laboratory |
| pUA93 | pGEX-TT-DLS1 | This laboratory |
| pUA135, 136, 137 | pGEX-TT-L1(WXXW), L2(AXXXW), L3(WXXWA) | This study |
| pUA141 | pRS315-DSL1<sup>W455A</sup>, LEU2, CEN6/ARS4 | This study |
| pUA143 | pGEX-TT-DLS1-Lc (aa 1–445) | This study |
| pUA151 | pQE30-RET2 | This study |
| pUA152 | pQE30-RET2c (aa 1–400) | This study |
| pUA157 | pGEX-TT-DLS1<sup>W455A/W495A</sup> | This study |
| pUA162, 162, 163 | pGEX-TT-DLS1-aa 1–200, aa 151–350, aa 301–449 | This study |
| pUA165 | pGEX-TT-DLS1-DN (aa 461–754) | This study |
| pUA168 | pGEX-TT-DLS1<sup>W441A/W451A</sup> | This study |
| pUA172, 173, 175, 173 | pGEX-TT-L4 (WXXW), L5(AAXXW), L6(AWXXXWA) | This study |
| pUA180 | pQE30-RET2N (aa 281–546) | This study |
| pUA183 | pRS315-DSL1<sup>W455A/W495A</sup>, LEU2, CEN6/ARS4 | This study |
| pUA188 | pRS315-DSL1<sup>W455A/W495A</sup>, LEU2, CEN6/ARS4 | This study |
| pUA190 | pGEX-TT-DLS1<sup>W441A</sup> | This study |
| pUA200 | pQE30-RET2-aa 191–400 | This study |
| pUA222 | Gal4p-DNA binding domain-DSL1<sup>W455A</sup>, URA3 | This study |
| pUA223 | Gal4p-DNA binding domain-DSL1<sup>W455A</sup>, URA3 | This study |
| pUA224 | Gal4p-DNA binding domain-DSL1<sup>W455A/W495A</sup>, URA3 | This study |

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**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. 2B shows that residues 444–459 of Dsl1p containing the δ-like WXXXW motif from Ds1p 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 δ-like motif (7), alanine substitution of either of the two tryptophan residues (W455A or W495A = AXXXW or WXXXXA; 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<sup>W455A/W495A</sup>) 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
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 δ-like WXXXW 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. 7A). At its C terminus this fragment contained an aspartic acid-rich region, which lies adjacent to the glutamic acid-rich δ-like 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- = WXW motif) and a third residue at position 425. To test the functional significance of the WXW 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 δ-like WXXXW-containing fragment (Fig. 2B). 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 (AXW) completely abolished the interaction of this Dsl1p fragment to δ-COP (Fig. 2). In summary, two di-aromatic motifs, WXW starting at position 413 and WXXXW at position 455, contribute to the binding of Dsl1p to coatomer.

To access the relative importance of the WXW and WXXXW motifs, single and double Trp → Ala substitutions (dsl1W413A, dsl1W455A, dsl1W413A/W455A) were introduced into full-length GST-Dsl1p. As mentioned above already, the W455A single substitution (AXXXW) did not significantly reduce the extent of binding to δ-COP (Fig. 3A). In contrast, the dsl1W413A mutant protein (AXW) showed a strongly reduced binding compared with the wild type. The double mutant (AXW...AXXXW) had the strongest effect and showed only negligible δ-COP binding in vitro. For this experiment, extracts of a δ-COP-overexpressing 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,
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. 3B 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/H11005 W455A/H11001 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 Trp3Ala 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 /H9254 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, /H9004 dsl1 W413A and /H9004 dsl1 W455A (A X W...A XXX W) showed growth on 5-FOA plates, suggesting that they can replace the wild type DSL1 gene very efficiently (Fig. 3C). In contrast, the /H9004 dsl1 W413A/W455A-expressing cells (A X W...) were not able to form colonies on these plates. The apparent lack of function of the double mutation was confirmed independently; /H9004 dsl1::kanMX expressing cells (AXW...AXXX) were not able to form colonies on these plates. The same plasmid was also unable to suppress the Ts –phenotype of /H9254 dsl1–22 mutant cells (data not shown) (32).

The single Trp → Ala substitutions (W413A or W455A =
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that the single mutants (32, 33). We examined BiP/Kar2p secretion by wild and the sec21 dsl1 mutants showed synthetic interaction with both the ret2–1 and the sec21–1 mutation (32). Together these results showed that the single ds11W413A and ds11W455A 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 ds11–22, ds11–4, and ds11–7 mutants (32, 33). We examined BiP/Kar2p secretion by wild type cells (DSL1), as well as ds11–22, ds11W413A, and ds11W455A 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 secretion extending to residue 280 reduced the interaction to the central part of Dsl1p (GST-L1; Fig. 2A). This indicates that the decrease in δ-COP binding of ds11 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-Ds1p was produced in E. coli and then immobilized on glutathione-Sepharose beads for binding experiments. His6-tagged versions of δ-COP produced in E. coli were applied to the GST-Ds1p-carrying beads. A truncation of δ-COP, which removed the last 146 residues, did not affect the binding in vitro (Fig. 4A, lane 6). However, an N-terminal deletion extending to residue 280 reduced the interaction to background levels (Fig. 4A, lanes 3 and 7). Lane 11 in Fig. 4A 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 (WXXW). 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. 2A; 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 (Asn, Gln, Tyr, Phe, Trp, Cys). We could confirm the results of the overlay assay by using peptides coupled to agarose. These peptides could bind GST-Ds1p, and they could recruit GST-L4 containing the WXW motif very efficiently from E. coli lysates. Fig. 4 (B and C)
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Ds1lp 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. As shown by Duden et al. (42), α-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/sec28Δ cells are viable when stabilized osmotically by the presence of 1 M sorbitol.2 The pull-down experiment shown in Fig. 6A (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. 6A, 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 mutant were used that also carried the ret2-1 defect (Fig. 6A, 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. 6A, lane 7). The cell extracts used for the pull-down samples shown in these last two lanes of Fig. 6A contained very little and mainly degraded coatomer (data not shown). However, the strong bind-

\[ \text{ret2} \text{ GST-Dsl1p when the panel} \]

\[ \text{cient as well as absence of rose. Fig. 6 were applied to GST-Dsl1p immobilized on glutathione Sepharose beads (B and D). A, immunoblot analysis of cell extracts from wild type cells and ret2Δ (δ-COP) cells expressing RET2 mutants carrying deletions in the putative Dsl1p-binding region, \( \Delta \text{A} \), \( \Delta \text{D} \), and \( \Delta \text{G} + \text{H} \). ΔA lacks residues 242–254, the stretch of residues, which gave the strongest and most specific signal in the overlay assay with GST-Dsl1p. The ΔD mutant carried an additional deletion of 14 residues (262–275). In mutant ΔG + H, 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. Transformers 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 ΔG + H 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. 2B). 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 ΔG + H mutation, aggravated this unusual behavior.

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2 H. D. Schmitt, unpublished observation.
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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 sec28Δ mutant (strain Y11469; e-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 sec28Δ cells (see above). This cross was performed to obtain the ret2-1sec28Δ double mutants. Double mutant cells from several tetrads had to be collected and grown in 1 M sorbitol medium to get enough starting material. sec28Δ 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 e-COP and ɛ-COP.

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 e-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 COPI (RET1) gene on 2-μm vector
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Fig. 7. Mapping of the binding site for Tip20p, α-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 sec28Δ 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-Dsl1pW143A/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- or α-ε-COP-overproducing cells had grown exponentially or were at stationary phase.

The results presented in Fig. 7A 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. 3A) 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 W143A/W455A double mutation. The results of these assays indicated that the double mutation affects the binding of α-COP as well, but not as led to a considerable increase in the amount of α-COP in extracts (Fig. 6B, upper panel). 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. 6B (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. 6B). 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 sec28Δ 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. 7A 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. 7A 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 experimental procedures were described above. Here they may serve as an internal control. A, 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- or α-ε-COP-overproducing cells had grown exponentially or were at stationary phase.

The results of the 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- or α-ε-COP-overproducing cells had grown exponentially or were at stationary phase.
isolated the DSL1 screen with Dsl1p is involved in this interaction. Our results indicate that the N terminus of Tip20p, a component of the membrane fusion machinery, is necessary to prevent binding of Dsl1p (32). The alignments of fungal Dsl1 proteins and the ZW10 proteins show high complexity region mediating coatomer binding is unique to fungi.

strongly as that of δ-COP (Fig. 7B). 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. 7C). In most experiments only very little α-COP from α/ε-COP-overexpressing cells interacted with GST-L1 (WXXXW), 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 (WXW) could recruit enough α-COP from the cell lysate to be seen by Coomassie staining. Surprisingly, the α-COP could still bind to the αXW 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.

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.

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 (WXXXXW) very much resembles the δl motif found by Cosson et al. (7). However, mutational analysis showed that a di-aromatic motif (WXW) 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. 7C indicated that the interaction with α-COP also involves a tryptophan residue that lies between the WXW and WXXXW 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 WXXXX 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 ascomycete fungus, for which the whole genome is known (48), may be unique among
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. 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 WxxxW 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

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3 M. Essid and H. D. Schmitt, unpublished observation.
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binding to both α-COP and δ-COP although to varying extents (Fig. 7B). In contrast, δ-COP mutants affected in Ds1p binding may be viable because Ds1p can still interact with α/δ-COP. To determine which interaction is more important, it will be necessary to identify the target site of Ds1p in α-COP. In fact, Cosson et al. (7) presented evidence that Ds1p as Part of a Putative Tethering Complex—Binding of Ds1p 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). Ds1p 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 Ds1p-Tip20p interaction because Ds1p 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 Ds1p the binding site for coatomer and Tip2p do not overlap, and Tip20-myc binds to GST-Ds1p with equal efficiency regardless of whether large amounts of α-COP were bound to it or not. This suggests that a simultaneous interaction of Ds1p 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 Ds1p acts as a direct link between a docking and a coat complex.

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