The EMBO Journal Vol.17 No.23 pp.6863–6870, 1998

New COP1-binding motifs involved in ER retrieval

Pierre Cosson[1], Yaya Lefkir, Corinne Démollière[2] and François Letourneur[3]

Institut de Biologie et de Chimie des Protéines, IBCP/UPR412 CNRS, 7 passage du Vercors, 69367 Lyon cedex 07, France, [1]Centre Médical Universitaire, Département de Morphologie, 1 rue Michel Servet, CH-1211 Genève 4 and [2]Basel Institute for Immunology, Grenzacherstrasse 487, Postfach CH-4005, Basel, Switzerland

[3]Corresponding author
e-mail: f.letourneur@ibcp.fr

Coatomer-mediated sorting of proteins is based on the physical interaction between coatomer (COP1) and targeting motifs found in the cytoplasmic domains of membrane proteins. For example, binding of COP1 to dilysine (KKXX) motifs induces specific retrieval of tagged proteins from the Golgi back to the endoplasmic reticulum (ER). Making use of the two-hybrid system, we characterized a new sequence (δL) which interacts specifically with the δ-COP subunit of the COP1 complex. Transfer of δL to the cytoplasmic domain of a reporter membrane protein resulted in its localization in the ER, in yeast and mammalian cells. This was due to continuous retrieval of tagged proteins from the Golgi back to the ER, in a manner similar to the ER retrieval of KKXX-tagged proteins. Extensive mutagenesis of δL identified an aromatic residue as a critical determinant of the interaction with COP1. Similar COP1-binding motifs containing an essential aromatic residue were identified in the cytoplasmic domain of an ER-resident protein, Sec71p, and in an ER retention motif previously characterized in the CD3ε chain of the T-cell receptor. These results emphasize the role of the COP1 complex in retrograde Golgi-to-ER transport and highlight its functional similarity with clathrin–adaptor complexes.

Keywords: COP1/endoplasmic reticulum/protein sorting/secretory pathway/T-cell receptor

Introduction

In eukaryotic cells, the membrane compartments of the exocytic and endocytic pathways are traversed by a constant flow of lipids and proteins (Rothman and Wieland, 1996; Schekman and Orci, 1996). The maintenance of the individual composition of each compartment therefore necessitates a precise sorting of membrane components at each step during intracellular transport. For example, during the formation of clathrin-coated endocytic vesicles, receptors destined to be endocytosed are concentrated in the forming vesicles, while others are excluded. This is due to the presence in the cytoplasmic domain of endocytosed receptors of specific YxxL endocytosis motifs which interact with components of the clathrin–adaptor complex (Kirchhausen *et al*., 1997).

Things are less clear in the case of coatomer (COP1)-coated vesicles, largely because there is still uncertainty about which steps of intracellular transport involve the COP1 complex (Bednarek *et al*., 1996; Cosson and Letourneur, 1997). COP1 is a protein complex composed of seven subunits α-, β-, β′-, γ-, δ-, ε- and ζ-COP, which forms a coat around vesicles budding from the Golgi apparatus (Rothman and Orci, 1992). The interaction of COP1 with dilysine (KKXX) motifs results in the retrograde transport of dilysine-tagged proteins back to the ER, suggesting that COP1 plays a key role in retrograde Golgi-to-ER transport (Cosson and Letourneur, 1994; Letourneur *et al*., 1994; Cosson *et al*., 1996). However, COP1 might also be involved in various other steps of intracellular transport, such as forward ER-to-Golgi and intra-Golgi transport. In particular, a recent study (Orci *et al*., 1997) suggests that two types of COP1-coated vesicles form at the level of the Golgi apparatus, some of which contain forward-directed cargo (proinsulin and VSV G protein), while others contain retrograde-directed cargo (KDEL receptor). It is noteworthy that another type of motif, the FF motif, might interact with COP1 and increase forward ER-to-Golgi and intra-Golgi transport of FF-tagged proteins (Fiedler *et al*., 1996). It is not known how the same COP1 protein complex can participate in diverse transport steps and interact with different motifs at each step.

Here we report the identification of a new family of COP1-binding motifs unrelated to dilysine or FF motifs, and we analyze its effect on the transport of tagged proteins in the early secretory pathway. These results illustrate the role of COP1-mediated sorting in the secretory pathway.

Results

Identification of a new COP1-binding motif

The δ subunit of the COP1 complex (δ-COP) presents a significant homology with medium (μ) chains of the clathrin-associated adaptor complexes (Cosson *et al*., 1996). Together they form a family of proteins composed essentially of a conserved N-terminal domain (150 residues) followed by a more variable C-terminal sequence. The divergent C-terminal portion of the μ adaptor chains interacts physically with YxxL endocytosis signals present in the cytoplasmic domain of a subset of membrane proteins (Ohno *et al*., 1995). By analogy, the C-terminal domain of δ-COP might be in a position which allows it to interact with membrane proteins during the formation of COP1-coated vesicles. However, putative motifs that could bind to the C-terminal portion of δ-COP remain to be identified.

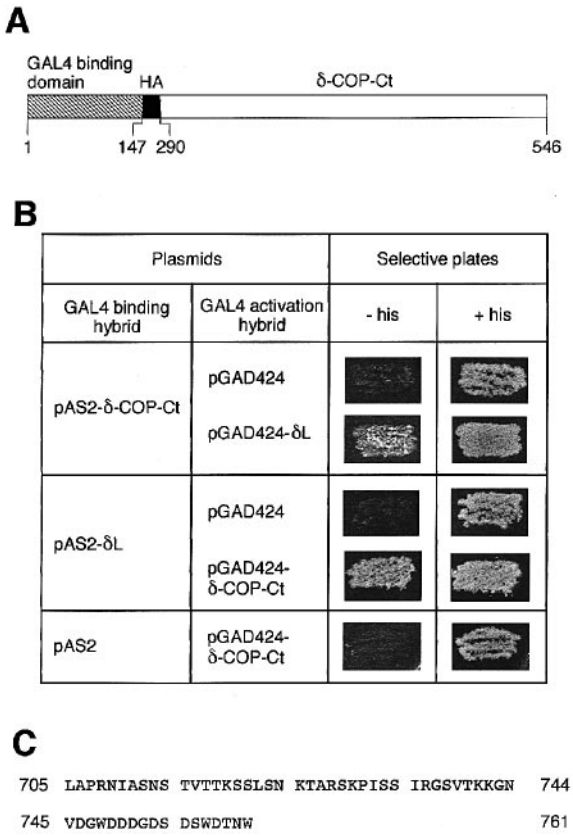

Fig. 1. Interaction of δ-COP with δ-COP ligand (δL). (**A**) Schematic representation of the construct used for the yeast two-hybrid screening. The sequence encoding residues 290–546 of δ-COP (δ-COP-Ct) was fused to the GAL4 DNA-binding domain sequence (GAL4BD) in the expression vector pAS2. (**B**) The *S.cerevisiae* reporter strain HF7c was cotransfected as indicated with plasmids encoding fusion proteins with the GAL4-binding domain or the GAL4-activating domain. Transformants were plated on synthetic medium without (–His) or with (+His) histidine. The sequence interacting with δ-COP was called δ-COP ligand (δL). (**C**) Peptide sequence of δL deduced from the cDNA insert of pGAD424-δL. This sequence corresponds to the C-terminus of a yeast protein of unknown function, labeled YOR112Wp in the SGD (DDBJ/EMBL/GenBank accession No. Z75020).

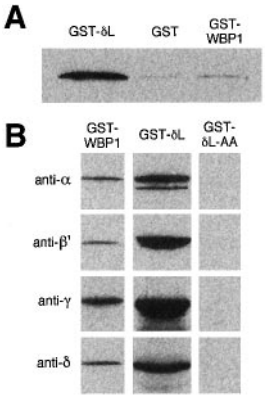

Fig. 2. *In vitro* binding of COP1 to δL. (**A**) Interaction between δL and δ-COP was tested *in vitro*. A fusion protein of δL with GST (GST–δL) was expressed in bacteria and immobilized on Sepharose–glutathione beads. It was then incubated with purified recombinant δ-COP tagged with (A) an N-terminal six-histidine sequence (His_6–δ-COP) or (**B**) with a whole-yeast-cell lysate. Proteins bound to the beads were separated on SDS–polyacrylamide gels, transferred to nitrocellulose and revealed (A) with antisera to δ-COP or (B) to various COP1 subunits, as indicated. In GST–δL–AA, the two C-terminal tryptophane residues of GST–δL were changed to alanine.

To isolate a protein sequence binding to the C-terminal domain of δ-COP, we made use of the two-hybrid system in yeast (Fields and Song, 1989). The sequence encoding residues 290–546 of yeast δ-COP (δ-COP-Ct) was fused to the sequence of the GAL4 DNA-binding domain (GAL4BD) in the expression vector pAS2 (Figure 1A; Harper *et al.*, 1993). In this assay, the formation of a complex between δ-COP-Ct fused to GAL4BD and a protein fused to GAL4AD should confer histidine auxotrophy and β-galactosidase activity. To screen for interacting sequences, yeast cells (HF7c) containing pAS2-δ-COP-Ct were transformed with an activation domain-tagged yeast genomic DNA library (GALAD). Transformants were plated on selective plates and subsequently tested for their ability to produce β-galactosidase using a filter assay (Breeden and Nasmyth, 1985). Library-derived plasmids were rescued and used to transform HF7c either alone or with three test fusions in pAS2 (data not shown). Out of 10 recovered plasmids, only one plasmid (pGAD424-δL) showed specific interaction with δ-COP-Ct. The sequence isolated by this two-hybrid screen was called δL, for δ-COP ligand. As shown in Figure 1B, the δL sequence that was fused either to the GAL4 DNA-binding domain (pAS2-δL) or to the GAL4 DNA activation domain sequences (pGAD424-δL) interacted specifically with δ-COP-Ct (expressed respectively in pGAD424 and pAS2), allowing growth of transfectants on selective plates. Measurement of β-galactosidase activity gave identical results (data not shown).

The cDNA insert of pGAD424-δL was sequenced and found to encode a peptide of 57 amino acids (Figure 1C). This sequence corresponds to the C-terminal end of a yeast protein of unknown function, labeled YOR112W in the Saccharomyces genome database (SGD; DDBJ/EMBL/GenBank accession No. Z75020). Computer-assisted protein sequence analysis predicted no potential transmembrane domain for this protein. Only an aminotransferase class-II pyridoxal-phosphate attachment site was detected (from a YPD protein search on YOR112W). Although the possibility was not formally discarded, no evidence was found for the native Z75020 protein being involved in intracellular transport in the early secretory pathway (see Discussion), and δL was used in this study as a protein sequence binding to δ-COP.

In vitro binding of COP1 to δL

To assess the specificity of the interaction between δL and δ-COP, a His_6 tag was transferred to the N-terminal end of δ-COP (His_6–δ-COP). The protein was expressed in *Escherichia coli* and purified on Ni-NTA resin. δL was also expressed in bacteria as a fusion protein with glutathione *S*-transferase (GST–δL). GST–δL was then immobilized on Sepharose–glutathione beads and incubated with purified recombinant His_6–δ-COP. While δ-COP did not bind to GST or GST–WBP1 (containing the dilysine motif of the yeast protein WBP1; Cosson and Letourneur, 1994), it bound efficiently to GST–δL in the absence of any cellular cofactors (Figure 2A).

To determine whether the fully assembled COP1 complex could bind to δL, whole-yeast-cell lysates were

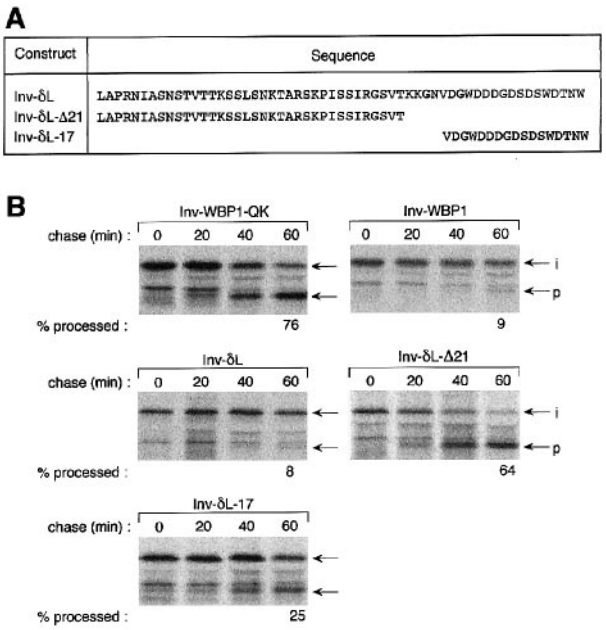

Fig. 3. δL induces ER localization of proteins. (**A**) Sequence of the peptides transferred to the reporter protein Inv-WBP1. (**B**) Yeast cells expressing the indicated invertase fusion proteins were metabolically labeled for 10 min and then chased for 0, 20, 40 or 60 min. Proteins were immunoprecipitated with an antiserum to invertase, treated with endoH and analyzed on SDS–polyacrylamide gel. The positions of intact (i) and vacuolar-processed (p) fusion are indicated by arrows. Percentage processed after 60 min was determined by PhosphorImager analysis and is also indicated. For the Inv–WBP1–QK construct, the second lysine residue of the dilysine signal was mutated to glutamine (Gaynor *et al.*, 1994).

incubated with GST–δL beads. Bound proteins were separated on SDS–polyacrylamide gels and revealed by immunoblotting with antisera to various coatomer subunits. The whole coatomer interacted with GST–δL beads as tested by immunoblotting with anti-sera to α-, β′-, γ- and δ-COP (Figure 2B), and to β- and ζ-COP (data not shown). ε-COP was not tested due to the absence of a specific reagent. Mutation of the two carboxy terminal tryptophane residues of δL to alanine (δL–AA) abolished COP1 binding (Figure 2B). In the conditions used here, COP1 bound even more efficiently to GST–δL than to GST–WBP1, which exhibits a dilysine motif (Cosson and Letourneur, 1994).

δL induces ER localization of tagged proteins in yeast cells

To assess the effect of COP1 binding on the intracellular transport of a membrane protein, we made use of a previously described reporter protein, Inv-WBP1, composed of the invertase protein fused to the transmembrane and cytoplasmic tail of WBP1 (Gaynor *et al.*, 1994). We substituted the dilysine sequence of Inv-WBP1 with the full-length δL sequence (Inv-δL), the last 17 residues of δL (Inv-δL-17) or with δL in which the last 21 amino acids were deleted (Inv-δL-Δ21; Figure 3A). Cells expressing invertase chimeras were pulse-labeled for 10 min and chased for 0 or 1 h. The fusion proteins were then recovered by immunoprecipitation with an antiserum to invertase, treated with endoglycosaminidase H (endoH) and resolved on SDS–polyacrylamide gels (Figure 3B). The fraction of the invertase chimeras that was processed

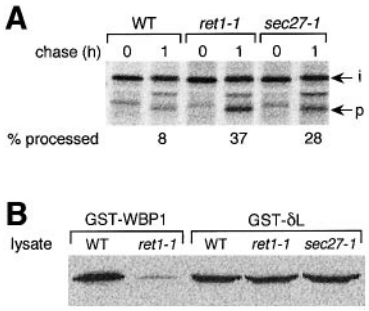

Fig. 4. ER retention of δL-tagged proteins is impaired in COP1 mutants. (**A**) Wild-type (WT, SEY6210), *ret1-1* (EGY101) and *sec27-1* (CKY100) cells expressing Inv-δL fusion proteins were analyzed as described in Figure 3. (**B**) Whole-cell lysates from the indicated cells were incubated with GST–WBP1 or GST–δL immobilized on glutathione–Sepharose beads. The adsorbed proteins were separated on SDS–polyacrylamide gels and revealed by immunoblotting with an anti-γ-COP antiserum.

in the vacuole after 1 h of chase was quantified by PhosphorImager analysis and is indicated. As previously reported, Inv-WBP1 is localized in the ER by virtue of the dilysine motif contained in the Wbp1p cytoplasmic domain, whereas mutation of the dilysine motif to QK (Inv-WBP1-QK) results in a protein that escapes to the vacuole and is proteolytically cleaved in a *PEP4*-dependent manner (Figure 3B; Gaynor *et al.*, 1994). Only 9% of the Inv-δL was processed after 1 h of chase, a rate comparable to that observed with Inv-WBP1. When the 17 residues of δL only were added to the invertase chimera, a significant though slightly less-efficient ER retention was observed. In contrast, deletion of the 21 C-terminal amino acids of δL resulted in efficient escape to the vacuole. Thus the δL COP1-binding sequence prevents escape of tagged proteins from the early secretory apparatus.

δL presents an ER retrieval determinant

Yeast coatomer mutants were previously reported to be defective for retrograde transport of dilysine-tagged proteins from the Golgi to the ER (Letourneur *et al*., 1994; Cosson *et al*., 1996). Inv-δL was expressed in various mutant cells, and its intracellular transport analyzed by pulse–chase at semi-permissive temperature (30°C) as described previously. Inv-δL expressed in *ret1-1* (α-COP) and *sec27-1* (β′-COP) mutant cells showed a significant vacuolar processing after 1 h of chase (37 and 28% of vacuolar processing, respectively; Figure 4A). A comparable rate of vacuolar processing was found for *ret2-1* (δ-COP), *ret3-1*(ζ-COP) and *sec21-1*(γ-COP) mutants (data not shown). These results demonstrated that ER localization of δL-tagged proteins was dependent on the function of the coatomer complex. As shown in Figure 4B, coatomer from *ret1-1* and *sec27-1* mutants was still able to bind GST–δL *in vitro* and this might account for the fact that the mislocalization of Inv-δL in COP1 mutants was less severe than observed with dilysine-tagged proteins (Letourneur *et al*., 1994).

The fact that COP1 is involved in ER localization of δL-tagged proteins suggested that the δL sequence could function as an ER retrieval determinant rather than a true ER retention determinant. In yeast cells, detailed analysis of the processing of *N*-linked sugars can provide information on the intracellular compartments reached by a

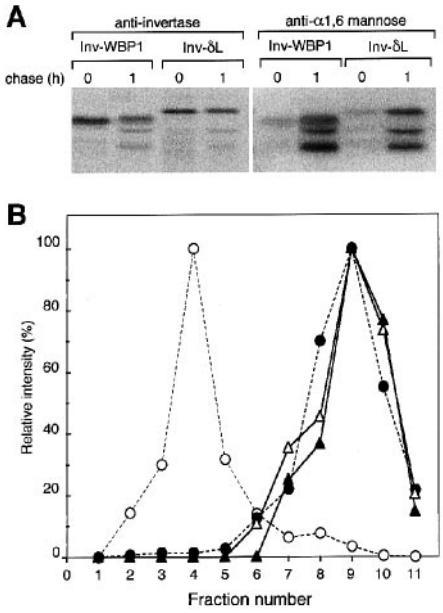

Fig. 5. The δL sequence is an ER-retrieval determinant. (**A**) To examine the mannose modifications of Inv–WBP1 and Inv–δL fusion proteins, cells expressing these constructs were labeled and chased as described in Figure 3. The fusion proteins were immunoprecipitated with an antiserum to invertase, eluted from the beads and reprecipitated with antibodies to α1,6 mannose. (**B**) Intracellular distribution of Inv-δL analyzed by velocity sedimentation on sucrose gradients. Yeast cells expressing Inv–δL were metabolically labeled for 20 min, then chased for 40 min, and the cleared lysate was loaded onto a sucrose gradient. Gradient fractions were collected (top, fraction 1; bottom, fraction 10; pellet, fraction 11) and an aliquot was analyzed by SDS–PAGE and immunoblotting using antibodies to an ER marker (Wbp1p, ●) or a Golgi marker (Emp47p; ○). Each fraction was then immunoprecipitated with an antiserum to invertase (▲). One aliquot of the immunoprecipitated material was eluted from the beads and reprecipitated with antibodies to α1,6 mannose (△). For each marker analyzed the relative intensity indicates the amount of signal relative to the maximum.

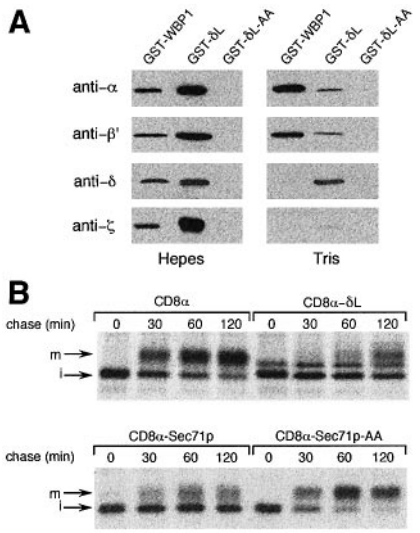

Fig. 6. The δL motif is functional in mammalian cells. (**A**) Binding of mammalian COP1 to δL. Lysates from COS cells were incubated with GST fusion proteins immobilized on glutathione–Sepharose beads as described in Figure 2. Bound proteins were separated on 8% SDS–polyacrylamide gels and revealed by immunoblotting with antisera to COP1 subunits as indicated. (**B**) COS-1 cells were transfected with plasmids encoding CD8α, CD8α-δL, CD8α-Sec71p or CD8α–Sec71p–AA (see text for details of the constructs). Cells were then metabolically labeled for 15 min, and chased for various times before lysis. The labeled CD8 proteins were isolated by immunoprecipitation and analyzed by SDS–polyacrylamide gel electrophoresis on 10% acrylamide gels under reducing conditions. The positions of the immature (i) and mature (m) CD8 are indicated.

glycoprotein. Addition of α1,6 mannose occurs in an early Golgi compartment, while elongation of α1,6-mannose sugar chains and addition of α1,3 mannose takes place in later Golgi compartments (Gaynor *et al*., 1994). Metabolically labeled proteins were immunoprecipitated with an anti-invertase antiserum, eluted from the beads, and reprecipitated with an anti α-1,6-mannose antiserum. After a 1 h chase, both Inv-WBP1 and Inv-δL could be reprecipitated with an antiserum to α1,6 mannose (Figure 5A), indicating that both fusion proteins have access to an early Golgi compartment. No acquisition of α1,3-mannose sugars was observed, indicating that both fusion proteins were restricted to the early secretory apparatus (data not shown). In *sec18* mutant cells at 37°C, in which vesicular transport from the ER to the Golgi is blocked, no acquisition of α1,6 or α1,3 mannose was observed (data not shown), confirming that the addition of these sugars is due to vesicular transport of Inv-δL fusion proteins to the Golgi apparatus.

To verify that Inv-δL is retrieved from early Golgi compartments back to the ER, we analyzed the intracellular localization of Inv-δL by subcellular fractionation. Metabolically labeled cells expressing Inv-δL were lysed and the supernatant of a 500 *g* spin was fractionated by velocity sedimentation on a sucrose gradient. The presence of Golgi (Emp47p) and ER (Wbp1p) markers in the gradient fractions was analyzed by immunoblotting (Figure 5B). Inv-δL was detected by immunoprecipitation with an antibody to invertase and one aliquot was further reprecipitated with antibodies to α1,6 mannose. Inv-δL cofractionated with Wbp1p (fractions 8–to 10) and was virtually absent in fractions containing Emp47p (fractions 3–5). Inv-δL from ER fractions was quantitatively reprecipitated with antibodies to α1,6 mannose (Figure 5B) but not with antibodies to α1,3 mannose (data not shown), demonstrating that after acquisition of α1,6 mannose in early Golgi compartments, Inv-δL is retrieved selectively to the endoplasmic reticulum.

δL binds mammalian COP1 and is functional in mammalian cells

Coatomer from mammalian cells partially disassembles at high ionic strength and partial complexes composed of the α, β′ and ε subunits were shown to bind dilysine motifs (Cosson and Letourneur, 1994; Lowe and Kreis, 1995). Under the same conditions, it was reported that partial complexes made of the β, γ and ζ subunits bind the FF motif found in the cytoplasmic domain in members of the p24 family (Fiedler *et al*., 1996).

GST–δL beads were incubated with whole COS-1 cell lysates prepared either in Tris (high ionic strength) or HEPES (low ionic strength) buffer as described previously (Cosson and Letourneur, 1994). Bound proteins were detected by immunoblotting with antisera to various COP1 subunits. In HEPES buffer α-, β′-, δ- and ζ-COP subunits bound to GST–δL and to GST–WBP1 (Figure 6A), as well as β-, γ- and ε-COP (data not shown), indicating that the fully assembled mammalian COP1 complex binds to

Table I. Characterization of COP1-binding motif in δL

Sequence	COP1 binding
VDGWDDDGDSDSWDTNW	+
DDDGDSDSWDTNW	+
DSDSWDTNW	–
VAAWDDDGDSDSWDTNW	+
VDGAADDGDSDSWDTNW	+
VDGWDAAGDSDSWDTNW	+
VDGWDDDAASDSWDTNW	+
VDGWDDDGDAASWDTNW	+
VDGWDDDGDSDAADTNW	–
VDGWDDDGDSDSWAANW	+
VDGWDDDGDSDSWDTAA	–
VDGWDDDGDSDSADTNW	–
VDGWDDDGDSDSWDTNA	–
VDGWDDDGDSDSADTNA	–
VDGWDDDGDSDSSDTNW	–
VDGWDDDGDSDSWDTNS	+
VDGWDDDGDSDSFDTNW	–
VDGWDDDGDSDSWDTNF	+
VDGWDDDGDSDSYDTNW	+
VDGWDDDGDSDSWDTNY	+
VDGWDDDGDSDSWDNW	+
VDGWDDDGDSDSWNW	–
VDGWDDDGDSDSWDTNWLGTFSSTN	+

Various mutations were introduced in GST–δL, and the resulting constructs tested for their ability to interact with COP1, as described in Figure 2. Binding of COP1 was assessed by immunoblotting with an anti-δ-COP antiserum. A positive binding corresponded to a signal of at least 25% of the control (GST fused to the 17 C-terminal residues of δ-COP).

δL. In Tris buffer, α and β′ subunits bound to GST–WBP1 beads, but not to ζ-COP, indicating effective dissociation of the complex. Under the same conditions, binding of all subunits to GST–δL with the exception of δ-COP was markedly reduced (Figure 6A and data not shown). These results indicate that δL does not bind efficiently to the αβ′ε or βγζ subcomplexes, and can thus be distinguished biochemically from the previously described dilysine or FF motifs.

To investigate whether the δL COP1-binding motif also functioned as an ER localization motif in mammalian cells, we constructed a chimera composed of the extracellular and transmembrane domains of the mouse CD8α fused to δL. As reported in previous studies (Hennecke and Cosson, 1993), after transfection of COS-1 cells, CD8α is transported efficiently out of the ER, as shown by conversion into Golgi-processed higher-molecular-weight species (Figure 6B). The substitution of the cytoplasmic tail of CD8α with δL resulted in a markedly slower exit of CD8–δL out of the ER (Figure 6B; 7% processing after a 30 min chase versus 67% for CD8α) demonstrating that binding of coatomer to δL can also induce ER localization of tagged proteins in mammalian cells.

The COP1-binding motif in δL

To identify the minimal sequence allowing an efficient binding of COP1, we constructed a series of GST fusion proteins in which only small stretches of δL were transferred (Table I). GST beads were incubated with whole-yeast-cell lysates and tested for COP1 binding by immunoblotting with an anti-δ-COP antiserum. Transfer to GST of the 17-amino-acid sequence VDGWDDDGDS-DSWDTNW was sufficient to induce efficient COP1 binding. However, deletion of residues DDDG within this sequence abolished the interaction, suggesting that negatively charged residues within this sequence could be necessary for binding of COP1.

The critical residues within the 17-amino-acid sequence were further delineated by scanning mutagenesis. Only mutations of either SW or NW to alanine residues abolished the binding of COP1. Single point mutations of both Ws to alanine demonstrated that only the first tryptophane residue was essential for COP1 binding, as substitution of the carboxy terminus W to S does not affect this binding. In addition, mutation of the C-terminal W to tyrosine or phenylalanine residues, two other aromatic residues, did not affect this interaction either. However, the mutation of the first W to tyrosine was the only permissive substitution (Table I). The deletion of two residues or the addition of four alanine residues between the two W of δL resulted in a strongly reduced COP1 binding showing that the spacing between the two W residues is important (Table I; data not shown). In contrast to dilysine signals, the positioning of the motif at the C-terminus is not essential. The addition of eight residues after this motif did not affect the interaction with COP1. In summary, the presence of an aromatic residue (position N) is the most essential feature of the COP1-binding motif of δL, but its environment is also important, and in particular the residue present at position N + 4.

A functional δ-COP-binding motif in Sec71p

Are δ-COP-binding motifs used to ensure retention of proteins in the early secretory apparatus? To assess this, we searched for potential motifs among proteins localized mainly in the endoplasmic reticulum. One possible candidate was Sec71p, a *Saccharomyces cerevisiae* integral ER protein required for translocation of secretory proteins in the ER lumen (Deshaies *et al.*, 1991). Its cytoplasmic domain (D^{147}SLQEAERLQPG<u>W</u>VQL<u>F</u>VMV^{166}) exhibits a potential δL motif preceded by some negatively charged residues. The wild-type cytoplasmic domain of CD8α was substituted with this sequence (Figure 6B). Its transport out of the ER was markedly slowed down (17% after 30 min), while mutation of the two aromatic residues to alanine restored efficient transport out of the ER (58% processing after 30 min; Figure 6B). These results suggest that a functional δL ER retrieval motif is present in the cytoplasmic domain of Sec71p.

A tyrosine-based ER localization motif in CD3-ε binds COP1

The CD3-ε chain of the T-cell receptor (TCR) is another protein localized in the ER by a motif containing an aromatic (tyrosine) residue. The TCR is a multisubunit receptor normally expressed at the plasma membrane of T cells. During its assembly in the ER, each unassembled subunit is degraded or retained in the ER (Klausner *et al.*, 1990). Mutagenesis studies have established that a tyrosine-containing motif comprising the 10-amino-acid sequence K^{171}GQRDLYSGL^{180} within the cytoplasmic tail mediates ER retention of unassembled CD3-ε

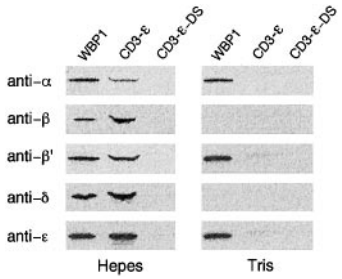

Fig. 7. The ER retention motif of CD3-ε binds to mammalian COP1. Lysates from CHO cells prepared in Tris or HEPES conditions were incubated with peptides coupled to Sepharose beads as indicated. Bound proteins were revealed as described in Figure 6 with antisera specific to mammalian COP1 subunits as indicated. WBP1 corresponds to the dilysine-containing peptide of Wbp1p (Letourneur *et al.*, 1994).

(Mallabiabarrena *et al.*, 1995). The mechanism of action of this motif remains unknown, and somewhat perplexing, as the presence of a YxxL motif is at first reminiscent of a clathrin-adaptor-binding endocytosis signal. Our results suggest that binding to coatomer could in fact account for the ER localization of proteins bearing this motif. Wild-type or mutant CD3-ε peptides (CI^{169}RKGQRDLY-SGLNQRRI^{185}, CI^{169}RKGQRDLDSGSNQRRI^{185}) were coupled chemically to Sepharose beads and incubated with whole CHO cell lysates prepared in Tris or HEPES buffer. Under HEPES conditions, all COP1 subunits bound specifically to CD3-ε peptides (Figure 7 and data not shown for γ- and ζ-COP). Mutation of ^{177}Y and ^{180}L to D and S, respectively, inhibited this binding. Dissociation of the COP1 complex in high-salt buffer (Tris buffer) prevented COP1 interaction to CD3-ε peptides as observed with GST–δL (Figure 7). The fact that COP1 must be fully assembled to bind CD3-ε peptides supported the conclusion that both δL and CD3-ε motifs belong to the same family of COP1-interacting motifs. Furthermore, a weak interaction of δ-COP with CD3-ε could be revealed by making use of the two-hybrid system (data not shown).

Discussion

In order to gain a better understanding of the role of coatomer in membrane sorting, we characterized a new motif which binds to the δ-COP subunit of the COP1 complex. The most striking feature of the δL (δ-ligand) motif is the presence of an aromatic residue, although the context of this residue is also important. No binding of the δL motif to COP2 (Bednarek *et al.*, 1996) was observed (data not shown). When transferred to the cytoplasmic domain of a reporter protein, the δL motif causes its continuous retrieval from early Golgi compartments back to the ER, thus ensuring its efficient localization in the early secretory pathway.

Though it is used in this study as a simple COP1 binding motif, the δL motif is originated from the open reading frame YOR112W, which encodes a yeast protein of unknown function. Sequence analysis predicted a cytosolic protein of 84.7 kDa. To test the function of this protein in the COP1-mediated retrograde pathway, we deleted the corresponding gene and followed the intracellular transport of three proteins: carboxy peptidase Y (CPY; a protein transported to vacuoles), Inv-WBP1 and Inv-δL (the latter two proteins normally retained in the ER). We did not detect any defects in secretion in null mutants (data not shown), suggesting that YOR112Wp is not essential for forward or retrograde transport in the early secretory pathway. We were also unable to coprecipitate COP1 and YOR112Wp in yeast cell lysates. Although we cannot formally exclude the possibility that the YOR112Wp is involved in intracellular transport, these results suggest rather that the δL motif present at its C-terminal end is not recognized by COP1 in the full-length protein.

Our results indicate that the Sec71p protein contains a potential δL motif in its cytoplasmic domain which could function as an ER retrieval signal *in vivo*. Indeed, Sec71p was recently shown to cycle continuously between the Golgi and the ER in a coatomer-dependent manner (Sato *et al.*, 1997). Although this study focuses on potential sorting motifs in the transmembrane domain of Sec71p, it leaves open the possibility that other ER retrieval motifs might participate in efficient ER retrieval of Sec71p. This issue is currently being addressed in our laboratory. Potential δL motifs can also be identified in the cytoplasmic domains of various other membrane proteins of the endoplasmic reticulum, such as the yeast proteins Alg1p (E^{429}AENRWQSNWER^{440}) or Pmt2p (E^{735}GPSSNFRYL-NWFSTWDIAD^{754}), which are mannosyltransferase and *O*-D-mannosyltransferase proteins, respectively (Couto *et al.*, 1984; Lussier *et al.*, 1995). In each case, it remains to be determined how important this putative motif is for the intracellular localization of the full-length protein.

Interestingly, we demonstrate that a previously identified tyrosine-containing motif that mediates ER retention of the unassembled CD3-ε subunit of the T cell receptor (Mallabiabarrena *et al.*, 1995) can also bind COP1. Only fully assembled COP1 complexes bind specifically to the CD3-ε motif, and this interaction is dependent on the presence of an aromatic residue within the motif. These data shed light on the role of COP1-mediated sorting in quality control of multisubunit complex assembly.

Together the results presented here argue in favor of an essential role of the COP1 complex in retrograde Golgi-to-ER transport and sorting of membrane components in this pathway. They also underline the functional similarity between the COP1 complex and the clathrin–adaptor complex. Indeed, the C-terminus of δ-COP must be in a position within the complex where it can interact with δL sorting motifs, a situation analogous to that of the μ chain of the clathrin-associated adaptor complexes interacting with YxxL motifs. The respective binding sites of δ-COP and of the μ chain of adaptor complexes might even be structurally related, as they both recognize similar sorting signals containing an aromatic residue. However, proteins with endocytosis motifs are evidently transported to the cell surface, suggesting that the motifs recognized by COP1 or clathrin adaptor complexes are distinct. Indeed, we could not detect any interactions between COP1 and GST fusion proteins comprising endocytosis tyrosine signals found in the cytoplasmic domains of the transferrin receptor or of TGN38 (data not shown; Ohno *et al.*, 1995). It is also possible that in some cases the same motif could be recognized first in the secretion pathway by COP1, then later at the plasma membrane by clathrin–adaptor proteins. The δL sorting motif of CD3-ε has been shown to adopt a helix–turn structure (Mallabiabarrena *et al.*, 1995). This structure is reminiscent of the β-turn structure

adopted by tyrosine-containing endocytosis signals. Therefore, the specificity of interaction should be provided by additional residues around the aromatic residues. The notion that ER retrieval motifs might also function as endocytosis motifs was previously proposed by Itin *et al.* (1995), who observed that a KKXX-related motif could target a membrane protein to the endocytic pathway. Further studies will be necessary to determine precisely the similarities and the differences between aromatic sorting motifs recognized by COP1 and clathrin coats, respectively.

Materials and methods

Strains, media and antibodies

The *S.cerevisiae* strain HF7c was provided by Clontech Laboratories Inc. (Palo Alto, CA) as part of a Matchmaker two-hybrid system kit. Yeast transformants were grown at 30°C in rich medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) or in synthetic minimal medium with appropriate supplements as described below. Yeast cells mutated in various coatomer subunits have been described previously (Letourneur *et al.*, 1994). COS-1 and CHO cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) medium with 5% FCS.

The rabbit antisera to various yeast COP1 subunits, Ret1p (α-COP) and Ret2p (δ-COP, No. 277), were raised by injection of peptides ^{1155}KPIYEDTPSVSDPLTGS^{1171} and ^{528}QELPYDVITSLKSDEYLVQ^{546}, respectively, synthesized on a MAP8 matrix (Bachem, Feinchemikalien Aktiengesellschaft). These antisera were both affinity purified on a column made of the same peptide coupled to activated CH Sepharose 4B (Pharmacia). The antiserum to Sec21p (γ-COP, No. 9256.9) and Sec27p (β′-COP, No. 9562) were a gift from R.Duden. Antisera to invertase and α1-6 mannose have been described previously (Gaynor *et al*., 1994). Antisera to mammalian α-, β-, β′-, γ-, δ-, ε- and ζ-COP were a gift from C.Harter/F.T.Wieland and J.Rothman. CD8α chimeras were detected using the 19.178 monoclonal antibody (Hämmerling *et al.*, 1979; Hennecke and Cosson, 1993).

Two-hybrid screen

The DNA sequence encoding residues 290–546 of δ-COP, referred to as δ-COP-Ct, was fused to the GAL4 DNA-binding domain sequence (GAL4BD) in the expression vector pAS2 (a gift of S.J.Elledge; Harper *et al.*, 1993). To screen for interacting proteins, yeast cells (HF7c) containing pAS2-δ-COP-Ct were transformed with an activation-domain-tagged yeast genomic DNA library (GAL4AD, Clontech Laboratories, Inc., Palo Alto, CA). Transformants were placed on selective plates (synthetic complete minus histidine, leucine and tryptophane, supplemented with 25 mM 3-aminotriazole). After selection, transformants were screened for their ability to produce β-galactosidase, using a filter assay (Breeden and Nasmyth, 1985). Library-derived plasmids were rescued and used to transform HF7c either alone or with three test fusions in pAS2 (data not shown).

Genetic techniques

Escherichia coli strain DH5α was used for plasmid propagation and purification. Plasmid DNA was purified using the alkaline lysis method (Maniatis *et al.*, 1982). Procedures for transformation of DNA into yeast (Becker and Guarente, 1991) and *E.coli* (Maniatis *et al.*, 1982) have been described previously. DNA sequencing was carried out using Sequenase (USB, Cleveland, OH).

Recombinant proteins

GST chimeras were prepared as described previously (Cosson and Letourneur, 1994). Briefly, yeast or COS-1 cells were lysed in Tris lysis buffer (50 mM Tris–HCl pH 7.6, 300 mM NaCl, 0.5% Triton X-100) or in HEPES lysis buffer (50 mM HEPES pH 7.3, 90 mM KCl, 0.5% Triton X-100) and the lysates applied to GST fusion proteins bound to glutathione–Sepharose (Pharmacia). Beads were washed in Tris or HEPES buffers containing 0.1% Triton X-100. Proteins were then separated on an 8% SDS–polyacrylamide gel, and this was followed by immunoblotting detection.

To test the interaction between δL and δ-COP, we tagged residues 290–546 of δ-COP with an N-terminus six-histidine sequence by cloning the corresponding sequence in pQE32 (Qiagen AG, Switzerland). The His_6–δ-COP protein was produced in *E.coli*, adsorbed on Sepharose–Ni beads (Qiagen AG, Switzerland) and eluted in 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.5 M imidazole and 1% Triton X-100. GST–δL Sepharose beads (50 µl) were then incubated with the purified His_6–δ-COP protein in 300 mM NaCl, 50 mM Tris pH 7.4 and 1% Triton X-100. After five washes in 150 mM NaCl, 50 mM Tris pH 7.4 and 0.1% Triton X-100, proteins were eluted by boiling and separated on a 10% SDS–polyacrylamide gel followed by immunoblotting detection with an anti-δ-COP antiserum (No. 277).

Cell labeling and immunoprecipitation

Analysis of the intracellular transport of invertase chimeras in yeast cells was performed as described previously (Gaynor *et al.*, 1994). Briefly, cells expressing invertase fusion proteins were pulse-labeled for 10 min with *Trans* ^{35}S-label and chased for 0 or 60 min. Equal amounts of cells were removed, and the fusion protein was recovered by immunoprecipitation with an anti-invertase antiserum, treated with endoH to remove *N*-linked oligosaccharides, and resolved on SDS–polyacrylamide gels. For reprecipitation, invertase chimeras were eluted from the beads at 90°C and subjected to a second round of immunoprecipitation with an antiserum to α1,6 mannose. Methods for SDS–PAGE (Laemmli, 1970), immunoblotting (Towbin *et al.*, 1979) and immunodetection by enhanced chemiluminescence (Amersham, Arlington Heights, Illinois) have been described previously.

For CD8 constructs, transfected COS-1 cells were labeled for 15 min at 37°C with 1 ml of 0.25 mCi/ml [^{35}S]methionine in methionine-free DMEM containing 5% fetal calf serum (FCS), as described previously (Hennecke and Cosson, 1993). Where indicated, labeled cells were chased by incubating at 37°C in DMEM containing unlabeled methionine and 5% FCS. At each time point, cells were scraped from the culture plate, pelleted by centrifugation and frozen at –70°C before detergent lysis in the presence of 10 mM iodoacetamide and immunoprecipitation. Immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions.

Subcellular fractionation

Subcellular fractionation by velocity sedimentation on sucrose density gradients was carried out as described previously (Schröder *et al.*, 1995). Briefly, cells (60 A_{600} units) were pulse-labeled with *Trans* ^{35}S-label for 20 min and chased for 40 min before adding 20 mM NaN_3 and 20 mM NaF. Cells were then converted to spheroplasts in a volume of 2 ml and lysed by at least 10 passages through a 25-gauge needle after adding protease inhibitors. The lysate was cleared twice for 5 min at 500 *g* in 2 ml reaction tubes. The supernatant (1 ml) was confirmed by microscopy to be devoid of unbroken cells and loaded onto an 11 ml sucrose gradient made up from 1 ml steps of 18, 22, 26, 30, 34, 38, 42, 46, 50, 54 and 60% (w/v) sucrose in 10 mM HEPES pH 7.5, 1 mM $MgCl_2$. The gradients were spun for 2 h 20 min at 4°C in a SW41ti rotor at 37 000 r.p.m. The gradient was then fractionated from the top into 12 equal fractions, the pellet being resuspended in the last fraction. Aliquots of the fractions were resolved by SDS–PAGE, transferred to nitrocellulose and probed with various antibodies as described.

In vitro binding of COP1 to CD3-ε

CI^{169}RKGQRDLYSGLNQRRI^{185} and CI^{169}RKGQRDLDSGSNQRRI^{185} peptides were synthesized (Eurogentech, Belgium) and coupled to activated thiol–Sepharose 4B (Pharmacia) according to the manufacturer's recommendations (5 mg of crude peptide per ml of beads). The coupling reaction was quenched for 1 h at room temperature in 0.1 M ammonium acetate pH 4, 0.5 M NaCl, 8.5 µM 2-mercaptoethanol and beads equilibrated and stored in PBS buffer. Binding to CD3-ε peptide beads and washing conditions were as described previously (Letourneur *et al.*, 1994).

Acknowledgements

We thank Alain Bosch and Christian Van Herrewege for artwork, and Michael Pepper for critical reading of the manuscript. P.C. is financed by a Start fellowship of the Fonds National Suisse de la Recherche Scientifique. The laboratory of FL is funded by the French Centre National pour la Recherche Scientifique (ATIPE), the Association pour la Recherche contre le Cancer and the Fondation pour la Recherche

Médicale. The Basel Institute for Immunology was founded and is financed by F.Hoffman-LaRoche and Company Ltd, CH4002 Basel, Switzerland.

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Received September 1, 1998;
revised and accepted September 25, 1998