Journal of Cell Science 111, 3459-3470 (1998)
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JCS0042

α-COP can discriminate between distinct, functional di-lysine signals in vitro and regulates access into retrograde transport

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Accepted 30 September; published on WWW 12 November 1998

SUMMARY

Emp47p is a yeast Golgi transmembrane protein with a retrograde, Golgi to ER transport di-lysine signal in its cytoplasmic tail. Emp47p has previously been shown to recycle between the Golgi complex and the ER and to require its di-lysine signal for Golgi localization. In contrast to other proteins with di-lysine signals, the Golgi-localization of Emp47p has been shown to be preserved in *ret1-1* cells expressing a mutant α-COP subunit of coatomer. Here we demonstrate by sucrose gradient fractionation and immunofluorescence analysis that recycling of Emp47p was unimpaired in *ret1-1*. Furthermore we have characterized three new alleles of *ret1* and showed that Golgi localization of Emp47p was intact in cells with those mutant alleles. We could correlate the ongoing recycling of Emp47p in *ret1-1* with preserved in vitro binding of coatomer from *ret1-1* cells to immobilized GST-Emp47p-tail fusion protein. As previously reported, the di-lysine signal of Wbp1p was not recognized by *ret1-1* mutant coatomer, suggesting a possible role for α-COP in the differential binding to distinct di-lysine signals. In contrast to results with α-COP mutants, we found that Emp47p was mislocalised to the vacuole in mutants affecting β′-, γ-, δ-, and ζ-COP subunits of coatomer and that the mutant coatomer bound neither to the Emp47p nor to the Wbp1p di-lysine signal in vitro. Therefore, the retrograde transport of Emp47p displayed a differential requirement for individual coatomer subunits and a special role of α-COP for a particular transport step in vivo.

Key words: Coatomer, Golgi, Endoplasmic reticulum

INTRODUCTION

One major means of protein transport through the secretory pathway is brought about by vectorial vesicular transport. Transport vesicles are generated by a variety of different proteinaceous coats which play a crucial role in the cargo selection, vesicle formation, and the delivery of the vesicle to the target membrane (Rothman and Wieland, 1996; Schekman and Orci, 1996).

Coatomer, a protein-complex consisting of seven different subunits (α-, β-, β′-, γ-, δ-, ε-, and ζ-COP) is the major building block of the coat of COP I-coated vesicles (Waters et al., 1991). COP I-coated vesicles may be involved in several different transport steps within the eukaryotic cell (Mallabiabarrena and Malhotra, 1995). First, COP I-coated vesicles have been described in anterograde transport of secretory proteins in the Golgi stack (Rothman, 1994; Orci et al., 1997). Second, they may be involved in ER to Golgi transport, a notion for which evidence from both mammalian and yeast cells has been collected. In mammalian cells antibodies against β-COP inhibit transport of VSV-G protein from the ER to the Golgi (Pepperkok et al., 1993), a process in which COP I-coated vesicles may act as a second carrier after COP II-coated vesicles in transport to the Golgi (Aridor et al., 1995; Rowe et al., 1996; Scheel et al., 1997). In yeast, it was demonstrated that COP I-coated vesicles form at the nuclear envelope in vitro (Bednarek et al., 1995) and some (but not all) yeast coatomer-mutants accumulate the ER-forms of some vacuolar and secretory proteins (Hosobuchi et al., 1992; Duden et al., 1994; Letourneur et al., 1994; Cosson et al., 1996; Wuestehube et al., 1996; Gaynor and Emr, 1997). In addition, coatomer is required specifically for the transport of GPI-anchored proteins from the ER to the Golgi (Sütterlin et al., 1997). Third, coatomer has clearly been shown to be essential for transport in the reverse direction, from the Golgi back to the ER (retrograde transport), in particular for proteins that carry a cytoplasmic di-lysine motif (Jackson et al., 1990, 1993; Gaynor et al., 1994; Townsley and Pelham, 1994; Lewis and Pelham, 1996; Teasdale and Jackson, 1996; Orci et al., 1997). Such di-lysine motifs bind coatomer in vitro, and mutants in coatomer subunits were identified by screening mutagenised yeast-cells for a defect in the ER-localization of a di-lysine reporter- protein (Cosson and Letourneur, 1994; Letourneur et al., 1994; Cosson et al., 1996). The fourth and most recently

proposed function of coatomer is in endosomal trafficking (Whitney et al., 1995; Aniento et al., 1996; Sheff et al., 1996; Daro et al., 1997).

How can one explain the various transport steps, in part in opposite directions, that COP I-coated vesicles may carry out and still fulfill the requirement for the maintenance of organelle identity? Part of the answer is likely to be found in the coatomer complex itself and the potentially variable function and assembly of its subunits. Coatomer from isolated endosomal fractions has been reported to contain relatively less γ- and δ-COP but more ζ-COP than Golgi-coatomer and to be differentially phosphorylated (Whitney et al., 1995; Sheff et al., 1996). Salt in high concentrations dissociates coatomer into subcomplexes in vitro (Cosson and Letourneur, 1994; Lowe and Kreis, 1995) which can still interact with di-lysine motifs (α-, β′-, ε-COP) or a novel di-phenylalanine motif (β-, γ-, ζ-COP) (Fiedler et al., 1996). However, direct evidence for differential function of coatomer subunits in vivo is still missing.

We have used Emp47p, a yeast type I transmembrane protein with a di-lysine signal, as a model protein to study retrograde transport from the Golgi to the ER. We have previously shown that Emp47p, unlike other known proteins with di-lysine signals, is located in the Golgi complex at steady state but can recycle to the ER. The steady state localization of the protein depends on its di-lysine signal, because a mutation of the signal leads to the escape of Emp47p from the Golgi to the vacuole (Schröder et al., 1995). The cytoplasmic tail of Emp47p comprising the di-lysine signal was also able to localise the α-factor receptor (Ste2p) to the ER, similar to previous observations with an α-factor receptor hybrid bearing the di-lysine signal of Wbp1p (Letourneur et al., 1994). Interestingly, while the α-factor receptor with the Emp47p di-lysine signal was no longer localised in the ER in a mutant affecting the α-COP subunit of coatomer (*ret1-1*), the steady state localization of Emp47p was unaffected. Here we analyse more comprehensively the effects of mutants in α-COP and other coatomer subunits on the retrograde transport of Emp47p. We provide evidence for at least two types of functionally distinct di-lysine signals, and provide the first in vivo evidence for a differential function of coatomer subunits on the localization of a particular di-lysine protein.

MATERIALS AND METHODS

Strains and growth conditions

Strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. Unless otherwise mentioned, strains were grown in YPUAD (2% yeast extract, 2% peptone, 40 mg/ml uracil and adenine, and 2% glucose) to exponential phase (0.5-2×10^7 cells/ml) at 30°C.

Generation of myc tagged Ste2p with the cytosolic tail of Emp47p

A myc epitope was inserted into the second extracellular loop of the previously described Ste2p-Emp47ptail construct (Schröder et al., 1995) by exchanging the *Hpa*I/*Cla*I fragment from the c-myc tagged complete receptor (Hicke et al., 1997).

Antibodies and indirect immunofluorescence

The two antisera against Emp47p AT (for 'anti-tail') and AL (for 'anti-lumenal') have been described previously (Schröder et al., 1995). The antibodies against the following antigens were used at the recommended dilutions: (A) polyclonal: Kar2p (kindly provided by R. Schekman, Berkeley, CA), Wbp1p (kindly provided by S. te Heesen, Zürich, Switzerland), Rer1p (kindly provided by H. D. Schmitt, Göttingen, Germany); coatomer (kindly provided by R. Duden, Cambridge, UK), α-COP (kindly provided by C. Harter and F. T. Wieland, Heidelberg, Germany); (B) monoclonal 9E10, directed against the myc-epitope (kindly provided by R. Movva, Sandoz AG, Basel, Switzerland). Indirect immunofluorescence was performed as described by Schröder et al. (1995).

Subcellular fractionation by sedimentation on sucrose density gradients

The procedure was essentially carried out as described by Schröder et al. (1995). Briefly, cells were grown in YPUAD to logarithmic phase, approximately 10^9 cells were harvested and resuspended in 10 ml YPUAD, cycloheximide was added to 20 μM and cells were then split into two aliquots which were incubated for 1 hour at either 24°C or 35°C after 5 minutes preincubation at room temperature. Intracellular ATP production was blocked by the addition of NaF and NaN_3 to 10 mM each. Cells were then converted to spheroplasts in 0.4 ml of a buffer containing 0.6 M sorbitol as osmotic support as described previously (Schimmöller et al., 1995) and osmotically lysed by the addition of an equal volume of distilled H_2O. The lysate was cleared at 500 ***g***. The resulting supernatant was confirmed by microscopy to be devoid of unbroken cells and was loaded onto a sucrose gradient (Schröder et al., 1995) and spun in a swing out rotor (TFT41.14; Kontron, Switzerland) at 4°C for 2.30 hours at 36K rpm. The gradient was then fractionated from the top into 13 equal fractions, the pellet being resuspended in the last fraction. Aliquots were resolved by SDS-PAGE, transferred to nitrocellulose and probed with the antibodies described above. Immunoreactivity was detected using the ECL kit (Amersham Int., Amersham, UK) and the ECL signal was quantified by scanning films with a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Generation of new *ret1*-alleles

New *ret1* alleles were isolated as previously described (Letourneur et al., 1994). Yeast cells expressing Ste2p-Wbp1p were mutagenized using ethylmethane sulfonate (50% cell death) and plated at high density on YPD plates. Three days later, they were replica plated to a lawn of *MAT*α cells, allowed to mate at 24°C for 6 hours, and replica plated to SD plates supplemented with histidine. The diploid cells that grew were sporulated and following tetrad dissection, ***MATa*** cells expressing Ste2p-Wbp1p and capable of mating were isolated. The different alleles were mapped by the allele recovery method (Rothstein, 1991) and the mutated genomic fragments directly sequenced by PCR.

Pulse-chase experiments

Analysis of the intracellular transport of the Inv-Wbp1p and CPY was performed as previously described (Letourneur et al., 1994). Briefly, cells expressing Inv-Wbp1 fusion protein (pEG1-KK) were preincubated for 10 minutes at the indicated temperature, pulse-labeled for 10 minutes with Trans ^{35}S-label and chased for 0 or 60 minutes. Equal amounts of cells were removed, and the fusion protein was recovered by immunoprecipitation, treated with endoglycosidase H to remove N-linked oligosaccharides, and resolved by SDS-PAGE. CPY was immunoprecipitated from the supernatant of the invertase immunoprecipitation and analyzed by SDS-PAGE. Pulse-chase experiments to determine the turnover of Emp47p were performed as follows. 2×10^7 cells per time point were harvested from a culture grown to log phase in YPUAD medium at 30°C. The cells were washed once with 15 ml of synthetic medium (1.7 mg/ml yeast nitrogen base, 5 mg/ml $(NH_4)_2SO_4$, 2% glucose, 30 μg/ml each leucine, lysine, tryptophan, histidine, uracil and adenine), resuspended and equilibrated in 2 ml synthetic medium for 15 minutes at 30°C. 2-

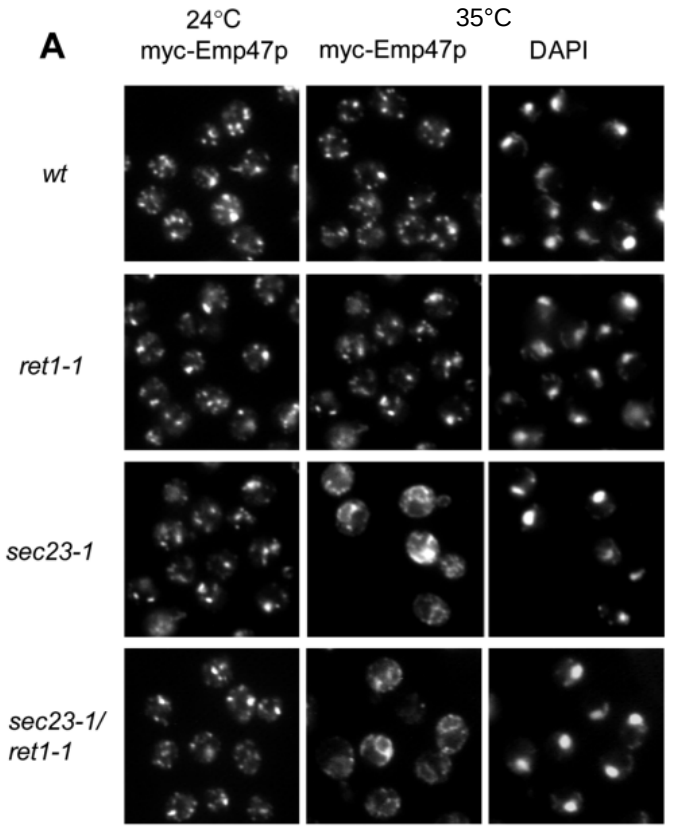
4 μl Trans^{35}S-label (18 mCi/ml, 1459 Ci/mmol, ICN, Eschwege, Germany) was added per time point and incubation was continued for 15 minutes. Cells were then harvested by a 2 minute centrifugation at room temperature and resuspended in 600 μl/time point prewarmed (30°C) YPUAD containing chase solution (3 mM ammonium acetate, 30 μg/ml each cysteine and methionine). Duplicate or triplicate aliquots were taken after the desired chase-times and transferred into prechilled tubes on ice containing NaN_3/NaF to yield a final concentration of 10 mM each. Cells were then either kept on ice until the last chase point or in an alternative protocol washed with cold PBS containing NaN_3/NaF and the pellets were frozen in liquid N_2. Cells were simultaneously converted to spheroplasts and lysed by incubation for 30 minutes at 30°C in 100 μl of a PBS solution containing 360 units lyticase/ml (Sigma, St Louis, MO), 100 mM β-mercaptoethanol, 10 mM each NaN_3/NaF, 5 mM EDTA, and one tablet EDTA-free protease inhibitors/6 ml (Boehringer Mannheim, Germany). Then 10 μl 10% SDS was added and the tubes were shifted to 95°C for 5 minutes. Insoluble material was removed by centrifugation and the clear supernatant was mixed with 1.5 ml TNET (100 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100). Emp47p was precipitated with AL antibody (Schröder et al., 1995) and Protein A-beads (UltraLink; Pierce, Rockford, IL) overnight at 4°C. Beads were washed with 4× 1 ml TNET and 3× 1 ml urea/SDS buffer (2 M urea, 0.1% SDS, 50 mM Tris-HCl, pH 7.6) prior to boiling in reducing sample buffer and loading for SDS-PAGE. The radioactive signals in the dried gels were recorded and quantified with a phosphorimager system (Molecular Dynamics, CA)

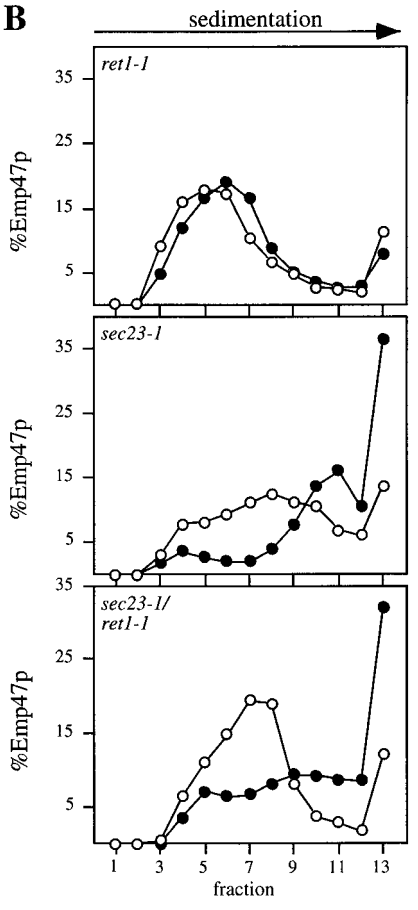
In vitro binding studies

Coding sequences for the cytoplasmic domains of Wbp1p (GST-WBP1 and GST-WBP1-SS; Cosson and Letourneur, 1994) and Emp47p (GST-Emp47, sequence: LIRQEIIKTKLL; GST-Emp47-SS, sequence: LIRQEIISTSLL) were cloned into the polylinker of the bacterial expression vector pGEX-3X (Pharmacia). GST fusion proteins were constructed by polymerase chain reaction mutagenesis (Higuchi et al., 1988). Yeast spheroplasts were prepared as described (Franzusoff et al., 1991) and lysed in Tris-Triton buffer (50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 0.5% Triton X-100, protease inhibitors, 1.8 mg/ml iodoacetamide, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride) (Manolios et al., 1990). Yeast lysates (5×10^{10} cells lysed in Tris-Triton buffer) were centrifuged for 15 minutes at 20,000 ***g***. Supernatants were incubated for 2 hours at 4°C (or otherwise indicated) with GST fusion proteins immobilized on Sepharose beads. Beads were washed once in Tris-Triton buffer, 4 times in wash buffer (50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 0.1% Triton X-100) once in phosphate buffered saline (PBS) and then eluted by boiling in reducing SDS sample buffer. Proteins were separated by SDS-PAGE (8% gels), transferred to nitrocellulose for immunoblotting as already described (Cosson and Letourneur, 1994) and revealed by enhanced chemiluminescence (Amersham, Arlington Heights, Illinois). When indicated the cells were lysed in Hepes-Triton buffer (50 mM Hepes, pH 7.3, 90 mM KCl, 0.5% Triton X-100, protease inhibitors) (Manolios et al., 1990). After incubation with the lysates, beads were washed 3 times in Hepes-Triton buffer and once in 50 mM Hepes, pH 7.3. Expression and purification of

Table 1. Yeast strains used in this study

Strain	Genotype	Source
EGY101	*MAT**a**, ret1-1, ura3, leu2, his3, trp1, suc2Δ9*	Letourneur et al., 1995
FLY76 (RH3445)	*MAT**a**, ret1-4, ura3, leu2, his3, lys2, ste2::LEU2*	F. Letourneur
FLY77	*MAT**a**, ret1-2, ura3, leu2, his3, lys2, ste2::LEU2*	F. Letourneur
FLY81 (RH3447)	*MAT**a**, ret1-5, ura3, leu2, his3, lys2, ste2::LEU2*	F. Letourneur
FLY89 (RH3449)	*MAT**a**, ret3-1, ura3, leu2, his3, trp1, suc2 Δ9*	F. Letourneur
PC130 (RH3440)	*MAT**a**, ret2-1, leu2, ura3, his, lys2, suc2 Δ9*	P. Cosson
PC133	*MAT**a**, ret2-1, leu2, ura3, lys2, his4, suc2Δ9*	Cosson et al., 1996
RH448	*MAT**a**, his4, leu2, ura3, lys2, bar1*	Lab strain
RH732	RH448 except *pep4::URA3*	Lab strain
RH1469	*MAT**a**, sec21-1, ura3, his4, leu2, lys2, bar1*	Lab strain
RH2047	RH1469 except *pep4::URA3*	Lab strain
RH2329	*MATα, leu2, ura3, his3, trp1, lys2, suc2Δ9*	Lab strain
RH2688	*MAT**a**, sec27-1, his4, ura3, leu2, bar1*	Lab strain
RH3187	*MAT**a**, ret1-1, ura3, leu2, trp1, his*	Lab strain
RH3274	RH3187 except *pep4::URA3*	Lab strain
RH3441	PC130 except *pep4::URA3*	This study
RH3446	FLY76 except *pep4::URA3*	This study
RH3448	FLY81 except *pep4::URA3*	This study
RH3453	RH1469 except *mycSTE2EMP47Ptail::URA3*	This study
RH3493	*MAT**a**, ret1-2, ura3, his, leu2, pep4::URA3*	This study
RH3494	*MAT**a**, ret1-2, ura3, his, leu2*	This study
RH3499	*MATα, ret1-1, trp1, ura3, leu2*	This study
RH3501	*MATα, sec23-1, ura3, leu2*	This study
RH3502	*MAT**a**, ret1-1, sec23-1, ura3, leu2*	This study
RH3505	FLY89 except *pep4::URA3*	This study
RH3515	RH448 except *mycSTE2EMP47tail::URA3*	This study
RH3516	PC130 except *mycSTE2EMP47Ptail::URA3*	This study
RH3517	FLY89 except *mycSTE2EMP47Ptail::URA3*	This study
RH3518	FLY81 except *mycSTE2EMP47Ptail::URA3*	This study
RH3519	FLY76 except *mycSTE2EMP47Ptail::URA3*	This study
RH3520	RH3494 except *mycSTE2EMP47Ptail::URA3*	This study
RH3521	RH2688 except *mycSTE2EMP47Ptail::URA3*	This study
RH3522	RH3187 except *mycSTE2EMP47Ptail::URA3*	This study
RH3603	*MAT**a**, sec23-1, trp1, his, ura3, leu2, mycEMP47::LEU2*	This study
RH3604	*MAT**a**, ret1-1, sec23-1, his, ura3, leu2, mycEMP47::LEU2*	This study
RH3605	*MATα, ura3, leu2, mycEMP47::LEU2*	This study
RH3606	*MATα, ret1-1, trp1, ura3, leu2, mycEMP47::LEU2*	This study
RH3609	RH2688 except *pep4::URA3*	This study





GST fusion proteins were performed as described (Smith and Johnson, 1988).

RESULTS

The retrograde transport of Emp47p is not affected by the *ret1-1* mutation

We previously demonstrated that the Golgi protein Emp47p contains a functional di-lysine ER-recycling signal and that mutations in this signal led to a rapid transport of the protein to the vacuole. Recycling of Emp47p could be demonstrated by trapping Emp47p in the ER upon block of export from the ER by using a temperature-sensitive *sec12* mutation (Schröder et al., 1995). The ER protein Sec12p is required for the formation of COP II-coated vesicles leaving the ER (Barlowe and Schekman, 1993). Unexpectedly the steady state localization of Emp47p was not affected in a strain with the mutant *ret1-1* allele of the gene coding for α-COP, the largest subunit of coatomer, even though the ER localization of a chimeric reporter protein consisting of the cytoplasmic tail of Emp47p fused to α-factor receptor (Ste2-Emp47ptail) was completely lost. This latter finding was similar to the behavior of an analogous fusion protein of Ste2p with the di-lysine signal of Wbp1p (Letourneur et al., 1994; Schröder et al., 1995).

The question remained then whether the failure of *ret1-1* to change the steady-state distribution of Emp47p reflected Ret1p-independent recycling of Emp47p. We attempted to address this question in a *sec12/ret1-1* double mutant, the rational being that if *ret1-1* would block retrograde transport, a redistribution of Emp47p into the ER should be abolished. For unknown reasons the *sec12/ret1-1* double mutants did not yield reproducible results. Therefore we assayed the effect of *ret1-1* on Emp47p recycling in combination with the *sec23-1* mutation. Sec23p is an essential component of the coat of COP II-coated anterograde ER transport vesicles (Hicke et al., 1992; Barlowe and Schekman, 1993; Yoshihisa et al., 1993) and has already been shown to trap recycling Emp47p in the ER (Lewis and Pelham, 1996). We grew cells at the permissive temperature of 24°C, added cycloheximide to avoid any contribution of newly synthesized Emp47p to the ER-signal and then either shifted the cells to the *sec23-1* nonpermissive

Fig. 1. Retrograde transport of Emp47p persists in *ret1-1* mutant cells. Cells were grown to log-phase in YPUAD at 24°C. After the addition of cycloheximide the cells were either kept at 24°C or shifted to 35°C for 60 minutes prior to immunofluorescence analysis (A) or sucrose density-gradient sedimentation analysis (B). (A) Wild-type (RH3605), *sec23-1* (RH3603), *ret1-1* (RH3606) and *sec23-1/ret1-1* (RH3604) with *myc-EMP47* integrated into the genome were fixed after incubation at the indicated temperatures and probed for myc-Emp47p. For the incubation at 35°C the DNA was visualised by DAPI staining. Each panel corresponds to 22 mm^2. (B) 10^9 cells of *sec23-1* (RH3501), *ret1-1* (RH3499) and *sec23-1/ret1-1* (RH3502) strains were osmotically lysed and the cleared lysates were loaded onto sucrose gradients. After centrifugation the gradients were fractionated into 13 fractions. Samples of each fraction were subjected to SDS-PAGE and blotted onto nitrocellulose filters. Emp47p was detected by AL-antibody and the resulting ECL-signals were quantified by scanning densitometry of exposed films. Open circles: 24°C; filled circles: 35°C.

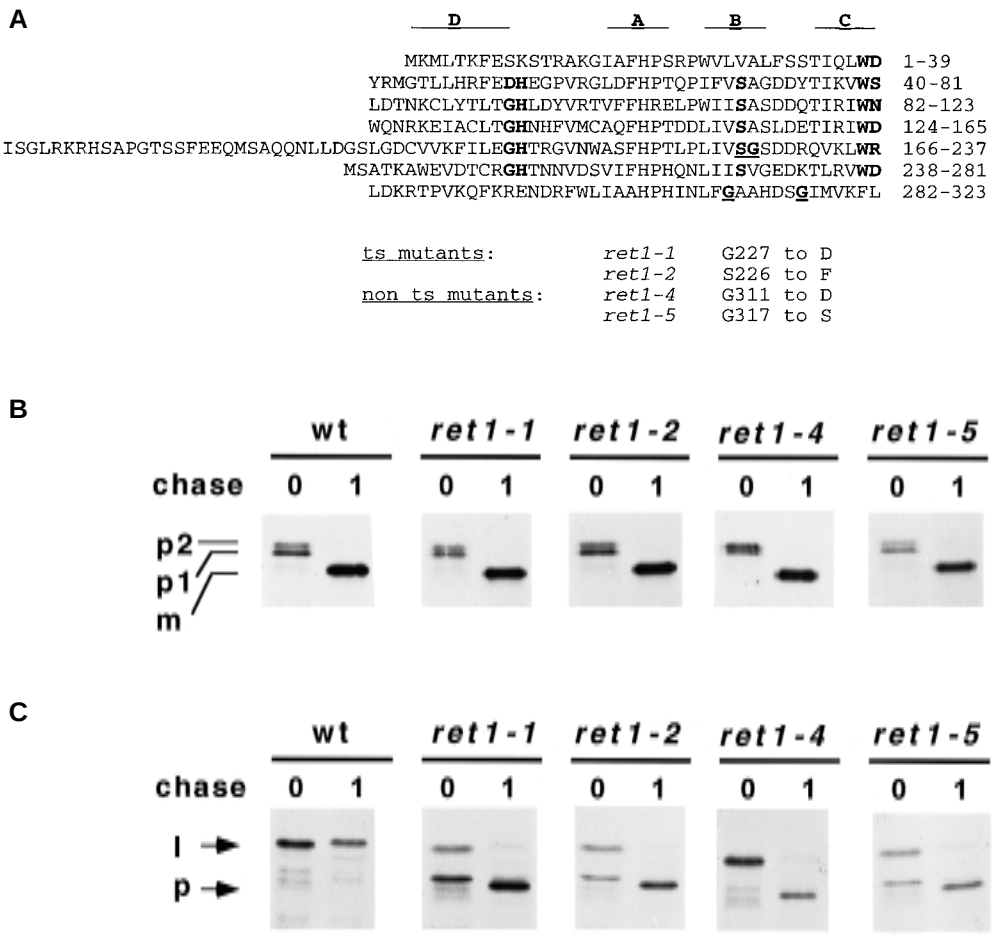


Fig. 2. Characterisation of three new alleles of *ret1*. (A) Sequencing of the *ret1* mutant alleles reveals that the mutations are in the N-terminal domain of Ret1p. The N-terminal domain of Ret1p comprising the six WD40 repeats with their four β-strands D, A, B, C (according to Wall et al., 1995) and a C-terminal extension up to amino acid 323 is depicted to line up the repeats. Amino acids conserved between the WD40 repeats of Ret1p and the repeats in the β-subunit of heterotrimeric G-proteins are printed in bold face. Amino acids that are mutated in the *ret1* alleles are bold and underlined. The alleles are classified according to ts or non-ts phenotype. (B) Pulse-chase analysis of the transport of CPY to the vacuole. Cells (wt, RH2329; *ret1-1*, EGY101; *ret1-2*, FLY77; *ret1-4*, FLY76; *ret1-5*, FLY81) expressing Inv-Wbp1 fusion protein were pulse-labeled for 10 minutes with Trans^{35}S-label and chased for 0 or 60 minutes at 30°C. Cell extracts were prepared and CPY was recovered by immunoprecipitation from the extracts. The protein was analysed by SDS-PAGE followed by autoradiography. The positions of the ER-core glycosylated form (p1), the Golgi-modified form (p2), and the vacuolar mature form (m) of CPY are indicated. (C) Pulse-chase analysis of the transport of Inv-Wbp1 fusion protein to the vacuole. Inv-Wbp1p was immunoprecipitated from the extracts described in B, N-linked glycans were removed by treatment with EndoH, and the protein was analysed by SDS-PAGE followed by autoradiography. Intact (I) and processed (P) fusion protein migrated at 70 and 56 kDa, respectively.

temperature of 35°C for 60 minutes or kept the cells at 24°C. Note that *ret1-1* is already defective for the retention of mycSte2-Emp47ptail at 24°C (data not shown). For immunofluorescence analysis of Emp47p recycling we integrated a myc-tagged version of Emp47p (Schröder et al., 1995) into the genome of the cells in order to achieve a more pronounced staining. As can be seen in Fig. 1A, at 24°C in *sec23-1* cells, myc-Emp47p was localised to punctate structures representing the yeast Golgi. At 35°C, myc-Emp47p was redistributed to the ER as visualised by the relocation of the fluorescence signal around the nucleus (compare to DAPI-stain). Wild-type cells retained their typical punctate Golgi pattern when stained for myc-Emp47p at both temperatures. In *ret1-1* cells, myc-Emp47p did not change its pattern upon shift to 35°C. However, in the *ret1-1/sec23-1* cells, Emp47p relocated to the ER, indicating that retrograde transport was still occurring in the double mutant.

The same effect could be observed using an independent experimental approach, the biochemical analysis of retrograde transport by differential sedimentation of microsomes on sucrose density gradients (Schröder et al., 1995). Fig. 1B shows the distribution of Emp47p on sucrose gradients which had been loaded with subcellular fractions obtained from *ret1-1-*, *sec23-1-* and *sec23-1/ret1-1-*cells incubated for 60 minutes at 24° or 35°C after the addition of cycloheximide. In *ret1-1* cells (Fig. 1B, top panel), Emp47p fractionated identically at 24°C and 35°C. Most of the Emp47p was located in the middle of the gradient where Golgi-membranes fractionate (Schröder et al., 1995), and a small amount sedimented to the bottom of the gradient, where ER is found (Schröder et al., 1995). In contrast, there was a clear difference in the fractionation properties of Emp47p in microsomes derived from *sec23-1* cells incubated at 24°C and 35°C respectively (Fig. 1B, second panel). The majority of Emp47p that was present in the middle of the gradient (i.e. Golgi) when cells were incubated at 24°C was shifted to the bottom of the gradient (i.e. ER) after the cells had been incubated at 35°C, indicative of retrograde transport. The same happened in the double-mutant *sec23-1/ret1-1* (Fig. 1B, third panel), confirming the result of the immunofluorescence analysis.

Characterization of three new *ret1*-mutant alleles

It is possible that the lack of effect of *ret1-1* on the recycling of Emp47p was due to a peculiarity of that allele. Therefore, we characterised three novel *ret1*-mutant alleles. These alleles were isolated by a genetic screen described previously (Letourneur et al., 1994) based on the mislocalization of a reporter protein consisting of the cytoplasmic tail of WBP1 (containing a di-

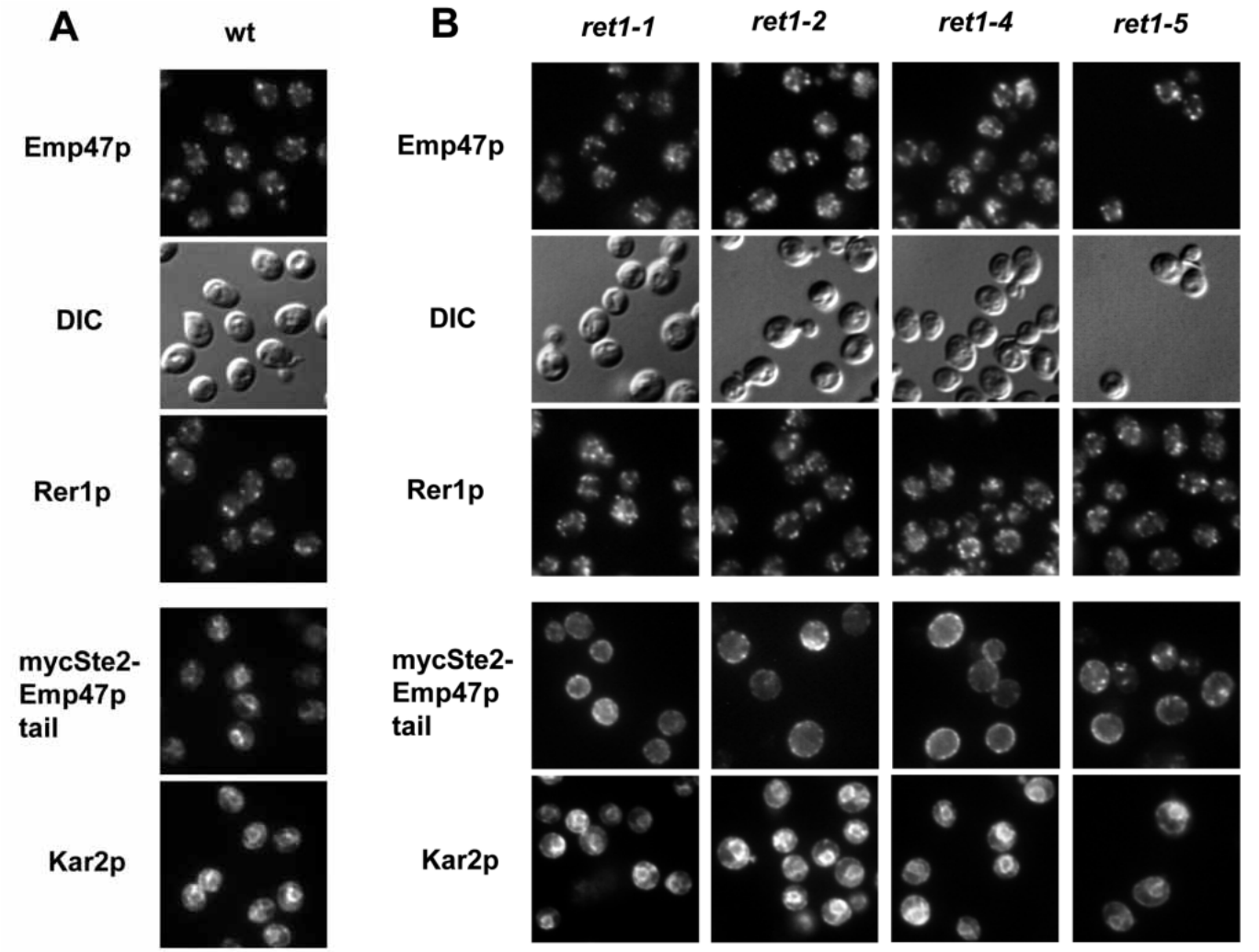


Fig. 3. Differential effect of *ret1* on the localization of two proteins with the same di-lysine signal in four *ret1*-alleles. Immunofluorescence analysis of the steady state localization of Emp47p and myc-Ste2-Emp47ptail in relation to other organellar markers. (A) Wild type, (B) *ret1* mutants. The cells in A and B are isogenic in each column with the following exceptions: the three uppermost panels are *pep4Δ* (wt, RH732; *ret1-1*, RH3274; *ret1-2*, RH3493; *ret1-5*, RH3448; *ret1-4*, RH3446). The cells in the two lowermost panels are *PEP4* and express mycSte2-Emp47ptail (wt, RH3515; *ret1-1*, RH3522; *ret1-2*, RH3520; *ret1-5*, RH3518; *ret1-4*, RH3519). Cells were grown at 30°C in rich medium prior to fixation. Note that the subcellular distribution of Emp47p was preserved in cells with mutant *ret1*-alleles, whereas mycSte2-Emp47ptail lost its ER-localization in all coatomer-mutants. Emp47p was detected by a mixture of AL- and AT-antibodies, Rer1p was detected by antiserum provided by H. D. Schmitt, Kar2p antiserum was provided by R. Schekman and mycSte2-Emp47ptail was detected with a-myc antibody 9E10. DIC, differential interference contrast. Each panel corresponds to 22 mm^2.

lysine signal) fused to the alpha factor receptor, Ste2p. We sequenced the mutant *ret1*-alleles and found the mutations to be located either in the fifth WD 40 repeat of Ret1p or in the immediate C-terminal vicinity of the sixth repeat (Fig. 2A). Interestingly two of the alleles (*ret1-4*, *ret1-5*) did not display a temperature-sensitive growth phenotype at 37°C (Fig. 2A). To assess a possible defect in the biosynthetic pathway in those mutants we analysed the transport of the vacuolar protease carboxypeptidase Y (CPY) to the vacuole. Cells were preincubated at the indicated temperatures for 10 minutes before a [^{35}S]methionine pulse labeling of 10 minutes. Cells were then chased for one hour and CPY was isolated by immunoprecipitation. None of the new alleles, *ret1-2* to *ret1-5*, had a strong anterograde transport defect as judged by the complete maturation of CPY within the chase-time (Fig. 2B).

Retrograde transport in the mutants was analysed by monitoring the escape to the vacuole of the di-lysine reporter protein, Inv-Wbp1, consisting of the entire invertase protein fused to the transmembrane domain and cytoplasmic tail of Wbp1p (Gaynor et al., 1994). The mislocalization of this chimeric protein to the vacuole becomes visible due to its vacuole-specific proteolytic cleavage product (Letourneur et al., 1994). Cells expressing Inv-Wbp1p were pulse-labeled for 10 minutes and chased for 0 or 60 minutes. The fusion protein was then recovered by immunoprecipitation, treated with endoglycosidase H (endoH), and resolved by SDS-PAGE. In contrast to wt-cells, all *ret1* alleles displayed nearly complete cleavage of Inv-Wbp1 within the chase period indicating a failure to recycle the reporter protein from the Golgi to the ER (Fig. 2C).

None of the *ret1* mutant alleles abolishes Golgi-localization of Emp47p

We then tested the influence of the novel *ret1*-alleles on the intracellular localization of various marker proteins by indirect immunofluorescence. The mutant cells along with wild-type controls were grown at 30°C, a permissive growth temperature for all of the *ret1*-mutants. Thus the following observations were made with viable cells. In order to be able to visualize a potential mislocalization of Emp47p to the vacuole we disrupted the *PEP4* gene coding for vacuolar proteinase A, preventing Emp47p degradation after arrival in the vacuole (Schröder et al., 1995). As can be seen in Fig. 3B, all *ret1*-alleles behaved indistinguishably for the markers analysed. Endogenous Emp47p yielded a punctate pattern comparable to that observed in wild-type cells (Fig. 3A). Only occasionally could one detect a faint vacuolar staining in the *ret1*-mutant cells upon comparison with the differential interference contrast (DIC)-images (Fig. 3B, vacuoles appear as indentations). This finding was corroborated by analysing the proteolytic turnover of Emp47p in pulse-chase experiments (Fig. 4). Cells which were *PEP4* were kept at 30°C throughout growth, pulse and chase. Aliquots were taken after the indicated chase-times and Emp47p was immunoprecipitated from the lysed cells. From the phosphorimager-analysis (Fig. 4) it is apparent that Emp47p turnover was slightly faster in *ret1-2*, *1-4* and *1-5* cells than in wild-type cells, but this was a marginal effect when compared to the accelerated turnover in *sec27-1* cells (Fig. 4) which will be further described below.

In order to assess Golgi-structure independently of Emp47p we assayed another Golgi-protein, Rer1p (Boehm et al., 1994; Sato et al., 1996; Boehm et al., 1997). We found that Rer1p colocalizes extensively with Emp47p. From a double immunofluorescence experiment 30 wild-type cells expressing myc-Emp47p were analysed. 150 Rer1p-positive fluorescent spots and 163 spots for myc-Emp47p were recorded, of which 121 spots were overlapping, amounting to 81% or 74% colocalization, respectively. Rer1p also yielded a punctate pattern in *ret1* mutant cells (Fig. 3B), providing additional evidence that the general Golgi-structure was not drastically altered in *ret1*-mutant cells.

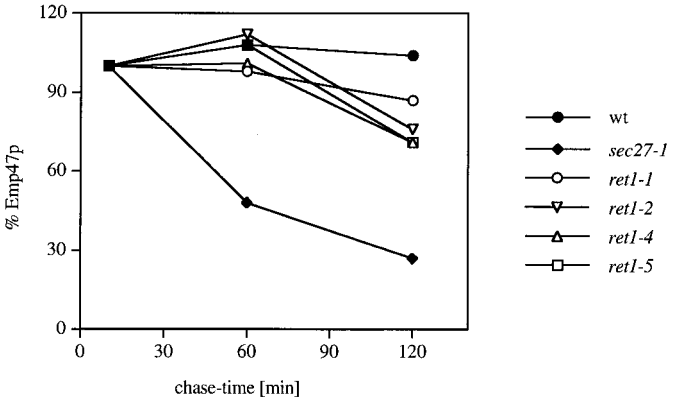


Fig. 4. Pulse-chase turnover of Emp47p in coatomer mutants. Wild-type (RH448), *ret1* mutant cells (*ret1-1*, RH3187; *ret1-2*, RH3494; *ret1-5*, FLY81; *ret1-4*, FLY76) and *sec27-1* cells (RH2688) were grown to log-phase, pulsed for 15 minutes with Trans^{35}S-label and chased for the indicated times, at which duplicate or triplicate samples were taken. After cell lysis Emp47p was precipitated with AL-antibody and the dried gels were analysed with a Phosphorimager system. Curves represent the average of three independent experiments.

A myc-tagged chimaera of Ste2p and the cytoplasmic tail of Emp47p (mycSte2-Emp47ptail) was localised to the ER in wild-type cells (Fig. 3A, row 4). It shows the same typical ER-fluorescence pattern as the yeast ER protein, Kar2p (Fig. 3A, row 5), most characteristically staining the nuclear envelope. As observed with Ste2-Emp47ptail in *ret1-1* cells (Schröder et al., 1995), the ER-pattern was completely lost for mycSte2-Emp47ptail in all *ret1* mutant strains. Instead we saw a peripheral staining, typical of plasma membrane (Fig. 3B, row 4). Note that the Kar2p-staining is identical in wt cells and in the *ret1*-mutants (Fig. 3B, row 5).

Emp47p is mislocalised to the vacuole in mutants affecting β´-, γ-, δ- or ζ-COP

The ability to localise Emp47p correctly in strains with different *ret1* alleles raised the question whether this observation would hold for mutants in other coatomer subunits as well. The immunofluorescence analysis (Fig. 5) shows that in cells carrying the mutant alleles *sec27-1* (β-COP), *sec21-1* (γ-COP), *ret2-1* (δ-COP) and *ret3-1* (ζ-COP), Emp47p could be visualized in the vacuoles of those cells grown at 30°C (compare DIC-images in Fig. 5). Partial mislocalization to the vacuole was already visible at 24°C (data not shown). We assayed the turnover of Emp47p in *sec27-1* cells in order to obtain a quantitative estimate of transport to the vacuole in comparison with the *ret1* alleles. We found that the apparent $t_{1/2}$ at 30°C in the *sec27-1* mutant was reduced to less than one hour (Fig. 4), close to the maximally possible value of about 50 minutes observed with an Emp47p variant lacking a functional di-lysine signal (Schröder et al., 1995). For *sec21-1* cells a drastically increased turnover of myc-tagged Emp47p has been reported (Lewis and Pelham, 1996).

In the non-*ret1* coatomer mutants the Golgi-protein Rer1p (see above) was still detected in punctate structures (Fig. 5, row 3). Thus the Golgi morphology was not grossly disturbed and the release of Emp47p showed specificity. Recently it was found that Rer1p itself recycles through the ER in a coatomer-dependent fashion (Boehm et al., 1997), although the recycling signal of Rer1p is unknown. However, Rer1p did not apparently leave the Golgi in an anterograde direction in any of the coatomer mutants.

MycSte2-Emp47ptail was mislocalised to the plasma-membrane in the non-*ret1* coatomer mutants (Fig. 5, row 4), equally to the *ret1* strains. Kar2p staining, however, constituted a second systematic difference between the *ret1* and the non-*ret1* coatomer mutants. In *sec27-1* and *ret2-1* cells especially, we observed that the Kar2p signal concentrated into spots (Fig. 5, row 5, compare Fig. 3A and B, row 5) reminiscent of BiP-bodies (Nishikawa et al., 1994). At a slightly higher temperature of 32°C similar spots containing Kar2p were also apparent in *sec21-1* and *ret3-1* cells (data not shown).

Coatomer from *ret1-1* cells but not from *ret2-1* cells binds to the cytosolic tail of Emp47p

Although we have shown that the di-lysine signal of Emp47p

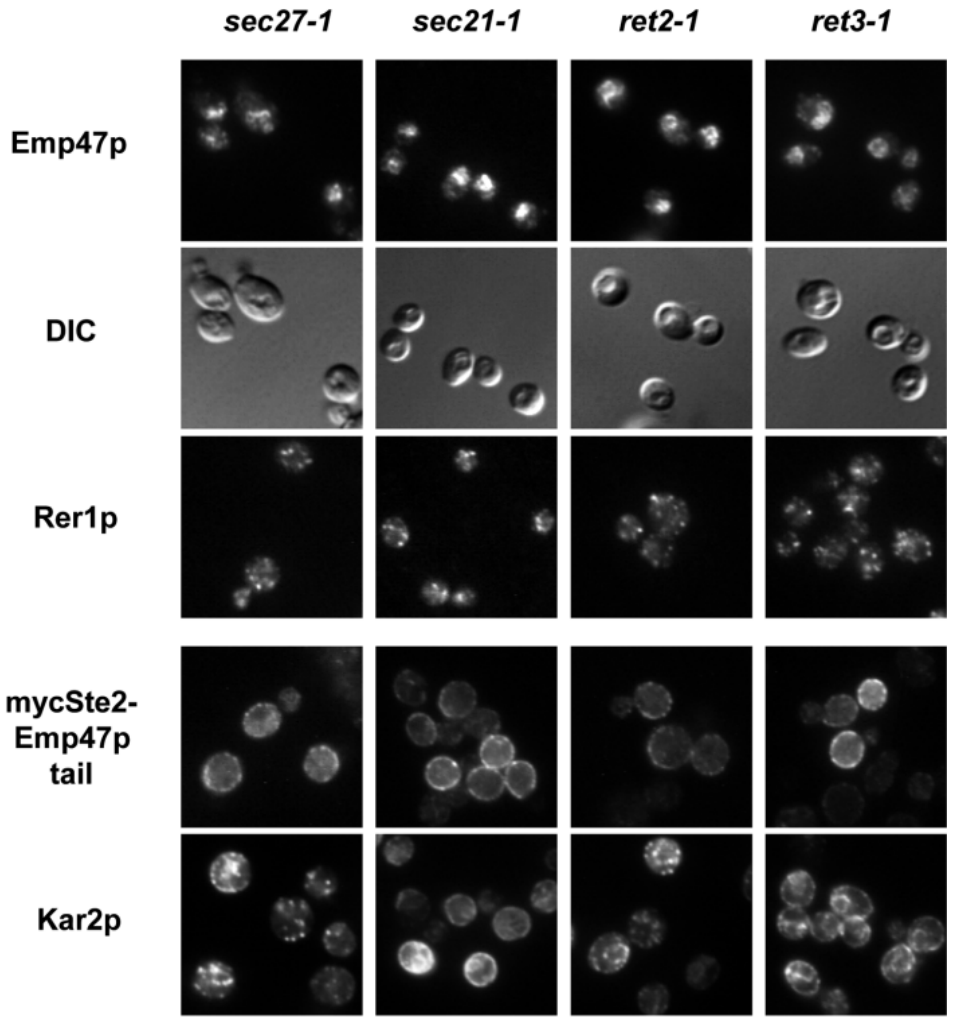


Fig. 5. Emp47p is mislocalised to the vacuole in coatomer-mutants other than *ret1*. Immunofluorescence analysis of the steady state localization of endogenous Emp47p and mycSte2-Emp47ptail in relation to other organelle-markers. The cells shown in each column are isogenic with the following exceptions: the three uppermost panels are *pep4Δ* (*sec27-1*, RH3609; *sec21-1*, RH2047; *ret2-1*, RH3441; *ret3-1*, RH3505). The cells in the two lower panels are *PEP4* and express mycSte2-Emp47ptail (*sec27-1*, RH3521; *sec21-1*, RH3453; *ret2-1*, RH3516; *ret3-1*, RH3517). Cells were grown at 30°C in YPUAD prior to fixation. Note that both Emp47p and mycSte2-Emp47ptail are mislocalised. Emp47p was detected by a mixture of α-lumenal and α-tail antibodies, Rer1p was detected by antiserum provided by H. D. Schmitt, Kar2p antiserum was provided by R. Schekman and mycSte2-Emp47ptail was detected with a-myc Antibody 9E10. DIC, differential interference contrast. Each panel corresponds to 22 mm^2.

is essential for Golgi localization and that it can be functionally transplanted onto a reporter-protein, direct evidence is missing to show that the tail of Emp47p interacts physically with coatomer. Therefore we performed in vitro coatomer binding experiments with yeast lysates from wild-type and coatomer mutant cells. Fig. 6A shows that a GST-fusion protein with the cytosolic tail of Emp47p was able to bind wt-coatomer as does the positive control, a GST-fusion protein with the tail of Wbp1p (Cosson and Letourneur, 1994). As with the Wbp1p-tail, the binding was dependent on the two lysine residues, because binding was abolished when these two residues were mutated to serine (lanes labeled S in Fig. 6A). All coatomer subunits were bound to Emp47p tail as tested by immunoblotting, excluding the possibility that only a subset of coatomer components binds to the Emp47p-tail. The binding efficiency was somewhat lower than with the Wbp1p-tail.

We next investigated whether there would be a correlation between the in vivo effects of coatomer mutants on Emp47p localization and the in vitro binding of coatomer. It has previously been found that coatomer from *ret1-1* cells was incapable of binding to the Wbp1p tail (Letourneur et al., 1994). When we performed the same experiment with the Emp47p tail we found, surprisingly, that coatomer from *ret1-1* cells could still bind to the Emp47p tail (Fig. 6B). This was true whether the binding reaction was performed at 4°C or at 30°C, the latter temperature being the one used to analyse Emp47p distribution in vivo. The picture was different with coatomer isolated from *ret2-1* cells which have a defective δ-COP subunit. Both Wbp1p-tail and Emp47p-tail bound *ret2-1*-coatomer at 4°C (Cosson et al., 1996), but not at 30°C. This in vitro binding behavior parallels the mislocalization (in *ret2-1*) or lack of mislocalization (in *ret1-1*) of Emp47p in the respective mutants in vivo. However, it does not help to explain the in vivo effects seen on the mycSte2-Emp47ptail hybrid, which is mislocalised in both *ret1-1* and *ret2-1*.

DISCUSSION

The protein complex coatomer is part of the sorting and transport machinery that is required for the retrograde Golgi to ER transport of di-lysine tagged- and other transmembrane proteins like Erd2p (Letourneur et al., 1994; Lewis and Pelham, 1996; Sonnichsen et al., 1996; Orci et al., 1997), Sec12p (Sato et al., 1996) and Rer1p (Boehm et al., 1997). In this work, we have shown that the retrograde transport of a particular di-lysine protein, Emp47p, was differentially affected by mutations in different subunits of coatomer.

Mutations in the β′-, γ-, δ- and ζ-COP subunits of coatomer led to a release of Emp47p from the Golgi to the vacuole to the same extent as an inactivation of the di-lysine sorting signal. All of these mutations also led to a loss of ER localization of the mycSte2-Emp47ptail and Invertase-Wbp1 reporter proteins. On the other hand, different mutations in the α-COP gene (*RET1*) completely mislocalised the mycSte2-Emp47ptail and Invertase-Wbp1 reporter proteins but did not strongly affect Emp47p localization (Figs 2B, 3B). Does this represent differential requirements for coatomer subunits in vivo or could the differential effects on Emp47p localization be simply related to the relative severity of the mutations (*ret1-n* 'weak', the others 'strong') with regard to a single function of coatomer?

All tested *ret1*-alleles released the mycSte2-Emp47ptail reporter to the plasma membrane indistinguishably from the other coatomer mutants, *sec27-1*, *sec21-1*, *ret2-1* or *ret3-1* (Fig. 3B, compare Fig. 5). Furthermore, *ret1-1* has an even more pronounced phenotype than other coatomer mutants in the mating-based assay which measures defects in the ER

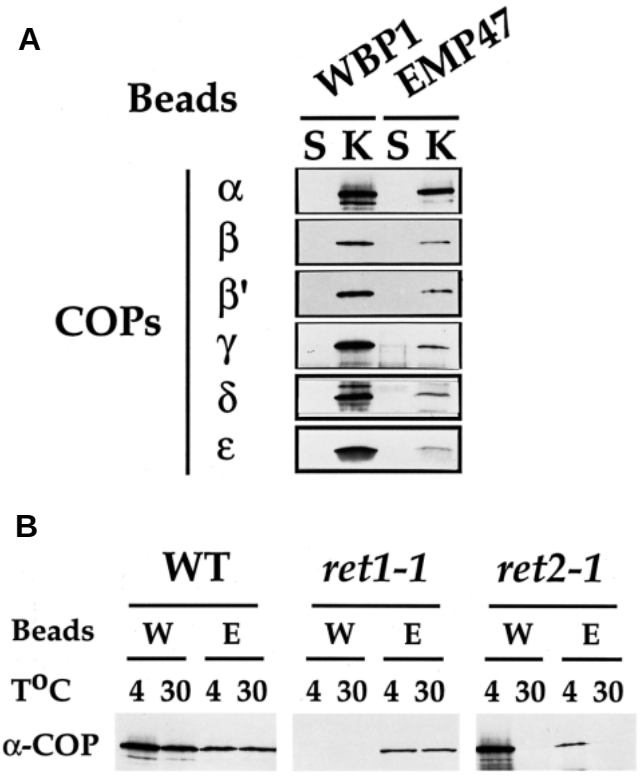


Fig. 6. Binding of coatomer to the di-lysine signal of Emp47p. (A) Whole wild-type cell lysate (RH2329) was incubated for 2 hours at 4°C with immobilized GST fusion proteins comprising the di-lysine signals of Wbp1p or Emp47p in either wild-type form (labeled K) or with a Lys to Ser mutation (labeled S). The absorbed proteins were eluted from the beads, separated by SDS-PAGE and the indicated coatomer subunits were revealed by immunoblotting using an anti-COP I antiserum (kindly provided by Dr R. Duden). (B) Cell lysates of wild type (RH2329) and *ret* mutant cells (*ret2-1:* PC133*; ret1-1:* EGY101) were prepared and incubated at 4 or 30°C as indicated on the figure with immobilized GST fusion proteins comprising the di-lysine signals of Wbp1p (W) or Emp47p (E). The adsorbed proteins were eluted from the beads and separated by SDS-PAGE. α-COP was revealed by immunoblotting using an α-COP antiserum.

localization of Ste2p tagged with the di-lysine-signal of Wbp1p (Letourneur et al., 1994; Cosson et al., 1996). Additionally, *ret1-n* strains mislocalize an invertase-di-lysine fusion protein to the vacuole much more efficiently than the other coatomer mutants (Fig. 3A, compare Cosson et al., 1996; Letourneur et al., 1994). Therefore, it is unlikely that the *ret1-n* alleles are simply weak mutations. It is more likely that the *ret1-n* strains behave in a divergent fashion from the other mutants with regard to Emp47p and other di-lysine tagged reporter proteins (Inv-Wbp1, Ste2-Wbp1, Ste2-Emp47ptail) due to a qualitative modification of coatomer function. Another systematic difference pointing to a special role of Ret1p in coatomer function is the distribution of the ER chaperone, Kar2p (yeast BiP). In *ret1-n* cells Kar2p localization was normal, while in the non-*ret1* mutants, Kar2p was localised to discrete spots similar to those described for various secretory mutants, among them *sec21* (Nishikawa et al., 1994). The reason for the formation of 'BiP-bodies' is not clear, but it may be interesting to note that BiP-body formation does not seem to correlate with the secretory defect of coatomer mutants because among the mutants with apparently no or only a minor ER to Golgi transport defect (*ret1-n*, *sec27-1*, *ret3-1*; Duden et al., 1994; Letourneur et al., 1994; Cosson et al., 1996) one finds mutants that form BiP bodies (*sec27-1*, *ret3-1*) and others that do not (*ret1-n*).

The molecular architecture of the coatomer complex(es) is only slowly emerging (Lowe and Kreis, 1995; Faulstich et al., 1996; Harter et al., 1996; Lowe and Kreis, 1996; Duden et al., 1998). Among the biochemical functions attributed to coatomer subunits are in vitro binding to membranes and receptor-tails (Cosson and Letourneur, 1994; Letourneur et al., 1995; Lowe and Kreis, 1995; Cosson et al., 1996; Fiedler et al., 1996). A subcomplex consisting of α-, β′-, ε-COP has been shown to bind to di-lysine signals in vitro (Cosson and Letourneur, 1994; Lowe and Kreis, 1995; Fiedler et al., 1996; Harter et al., 1996) whereas a β-, γ-, ζ-COP subcomplex has been claimed to bind a novel phenylalanine-containing signal in vitro (Fiedler et al., 1996).

Our in vitro binding experiments provide new pieces of information to the still rudimentary picture. Coatomer from *ret2-1* (defective in δ-COP) showed a temperature-dependent loss of in vitro binding to both Wbp1p-tail and to Emp47p-tail, despite the fact that an α-, β′-, ε-COP subcomplex devoid of δ-COP was still able to bind di-lysine signals. Therefore, mutant δ-COP may disturb the structure of the coatomer complex in a way that changes the binding properties of α-, β′-, ε-COP. Alternatively, multiple subunits, including δ-COP, could bind to di-lysine containing tails, perhaps being regulated by the specific nature and sequence context of the signal. This assumption may reconcile the finding that γ-COP can be crosslinked to di-lysine signals (Harter et al., 1996).

Our in vitro binding data of *ret1-1* mutant coatomer add a further level of complexity to the interaction of coatomer with di-lysine signals. While binding of coatomer from *ret1-1* cells to the Wbp1p-tail is abolished, the Emp47p-tail binds the *ret1-1* mutant coatomer with approximately the same efficiency as wild-type coatomer. Two possible interpretations of this result are: (1) If α-COP binds directly to di-lysine signals, then the signal must contain more information than the presence of the lysine residues. (2) If α-COP is not the (only) signal-binding subunit, it may be neccessary to regulate the

binding of other subunits to some types of di-lysine signals (like Wbp1p) but not others (like Emp47p).

Functional consequences of coatomer binding to di-lysine signals

The inability of coatomer from *ret2-1* and the ability of coatomer from *ret1-1* cells to bind Emp47p-tail correlates with the observation of mislocalization of Emp47p in vivo in *ret2-1* cells and the unaltered localization in *ret1-1 cells*. This suggests that di-lysine binding of coatomer may be required for retrograde transport of the target protein. On the other hand, mycSte2-Emp47ptail, presenting the same di-lysine signal, is equally mislocalised in *ret2-1* and *ret1-1* cells. It is clear already from the steady state distribution of Emp47p (Golgi) and mycSte2-Emp47ptail (ER) in wild-type cells, that the di-lysine signal alone cannot account for the different trafficking behavior of the two proteins. Many transmembrane proteins contain multiple features in their lumenal, transmembrane and cytoplasmic domains that ultimately determine their trafficking: e.g. composition and length of the transmembrane domain(s) (Bretscher and Munro, 1993; Munro, 1995), lumenal interactions via protein or glycan-determinants (Itin et al., 1995; Scheiffele, 1995), shielding (Letourneur et al., 1995) or covalent modification (Schweizer et al., 1996) of sorting signals, or presence of multiple cytoplasmic sorting signals (Itin et al., 1995; Kappeler et al., 1997). So it may not be surprising that two proteins as different as the plasma-membrane resident polytopic Ste2p and a type I transmembrane protein of the Golgi would behave differently in response to a signal for ER/Golgi localisation. It could be that either: (a) Ste2p contains additional anterograde information that overrides the recycling information in the *ret1-1* context; or (b) that Emp47p contains additional recycling information that keeps it recycling in *ret1-1* mutant cells, or (c) both. The short transmembrane-domain of Emp47p may facilitate the partitioning of Emp47p to the rims of the Golgi cisternae where COP I-coated retrograde vesicles bud, whereas the long transmembrane domains of Ste2p would increase the tendency to move in an anterograde direction through the Golgi and thus require a strong 'capturing' function of Ret1p. However, simply lengthening the single transmembrane-domain of Emp47p by 3 or 4 extra leucine residues apparently does not abolish its Golgi-localisation in *ret1-1* mutants (our unpublished observation). Also, in contrast to its mammalian homologue ERGIC-53 (Schweizer et al., 1988), Emp47p seems not to form disulfide linked oligomers (our unpublished observation), which may provide for multiple, cooperative binding sites sustaining recycling in *ret1-1*. Importantly, we may however derive from our experiments that a di-lysine signal that is competent to bind coatomer from α-COP mutant cells (*ret1-1*) appears to be necessary, but not sufficient to keep a molecule on the recycling pathway in the mutant cell.

All *ret1*-alleles described in this work (*ret1-1, 2, 4, 5*) code for mutations in Ret1p that are closely spaced within 85 of the 1,201 amino acids of the protein, either within (*ret1-1, 2*) or immediately adjacent (*ret1-4, 5*) to the WD40 repeats in the N-terminal region of Ret1p. WD40 repeats are structural elements found in many proteins, among them β′-COP (Stenbeck et al., 1993), Sec13p (Pryer et al., 1992) and Sec31p (Barlowe et al., 1994). In the β-subunit of heterotrimeric G-proteins seven WD40 repeats form a propeller-like structure with every repeat contributing one blade (Wall et al., 1995; Clapham, 1996; Sondek et al., 1996). Both the blades and the top and bottom surfaces of the protein interact with the other subunits of the G-protein. The conserved Ser/Thr-20 in a prototypic WD40 repeat is part of a hydrogen-bonded tetrad contributing to the stability of the propeller-blades (Wall et al., 1995). Ser226 in the fifth repeat of Ret1p corresponds to Ser/Thr-20 and is mutated to a Phe in *ret1-2*. The mutation in *ret1-1* is immediately adjacent, at Gly227. It is, however, not clear what effect these mutations have on the structure and function(s) of Ret1p and the whole coatomer complex. Importantly the ts-phenotype of *ret1-1* can be partially suppressed by the expression of a single copy-plasmid coding for an incomplete *RET1* gene that does not contain the WD40 repeats (Letourneur et al., 1994). It seems, therefore, that a mutation in the N-terminal WD40 repeat domain can exert a dominant *cis*-acting effect at high temperatures on an essential coatomer function mediated by the C-terminal portion of Ret1p. Such a function may be the interaction with ε-COP, which has been suggested to be disturbed in a very restrictive allele of α-COP, *ret1-3* (Duden et al., 1998).

In summary, our data show firstly that there are different types of di-lysine signals which can be distinguished in vitro by mutations in the α-COP subunit that differentially modify the binding properties of coatomer to these signals. Secondly, the binding of coatomer to these signals is likely to be required for retrograde transport, but the ability to bind these signals in vitro does not guarantee that a particular protein will be transported in the retrograde pathway. Our data also further support the crucial role of coatomer in the retrograde transport pathway. The future development of an in vitro transport assay from the Golgi to the ER should help us to investigate the proposed multiple functions of coatomer subunits in more detail.

We thank R. Duden, C. Harter, R. Movva, R. Schekman, H. D. Schmitt, S. te Heesen and F. T. Wieland for the gift of antibodies, Pierre Cosson for strains and discussion. Corinne Demolliere and Thomas Aust have helped us with excellent technical assistance. S.S.-K. thanks D. Gallwitz for generous support. This work was supported by the Canton of Basel-Stadt and a grant of the Swiss National Science Foundation to H.R., an EMBO long term fellowship to S.S.-K. and in part by a grant of the Deutsche Forschungsgemeinschaft to S.S.-K.

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