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# Coatomer Interaction with Di-Lysine Endoplasmic Reticulum Retention Motifs

Pierre Cosson and François Letourneur*

**Although signals for retention in the endoplasmic reticulum (ER) have been identified in the cytoplasmic domain of various ER-resident type I transmembrane proteins, the mechanisms responsible for ER retention are still unknown. Yeast and mammalian ER retention motifs interacted specifically in cell lysates with the coatomer, a polypeptide complex implicated in membrane traffic. Mutations that affect the ER retention capacity of the motifs also abolished binding of the coatomer. These results suggest a role for the coatomer in the retrieval of transmembrane proteins to the ER in both yeast and mammals.**

After translocation in the ER, proteins destined to the exocytic pathway are believed to be transported to the cell surface by default, unless retained in subcellular compartments by specific signals (*1*). For instance, a short KDEL (*2*) motif at the COOH-terminal end of soluble proteins can cause efficient retention in the ER (*3*). In the case of type I transmembrane proteins, ER retention motifs have been described in their transmembrane domains (*4*). Cytoplasmic ER retention motifs have also been identified in various mammalian proteins resident in the ER (*5*). Essential elements of these motifs are two lysine residues located at positions −3 and −4 from the COOH-terminal end of the cytoplasmic domain (*6*). Residency in the ER that is mediated by KDEL and di-lysine motifs is believed to be achieved by continuous retrieval to the ER of tagged proteins from post-ER compartments (*7*, *8*). The receptor for the KDEL signal has been cloned and is localized in post-ER compartments reached early in the transport process, where it can sort KDEL-tagged proteins and ensure their transport back to the ER. The molecular mechanisms for ER retrieval of di-lysine–tagged proteins are unknown.

WBP1 is an ER-resident transmembrane protein from yeast that is a component of the yeast *N*-oligosaccharyl transferase complex (*9*). Its cytoplasmic domain contains a di-lysine motif (Fig. 1A) that acts as an ER retention signal in yeast (*10*). To test whether the yeast di-lysine ER retention signal also functions in mammalian cells, we transferred the cytoplasmic domain of WBP1 to the COOH-terminus of the Tac antigen (T-WBP1), a mammalian type I membrane protein normally expressed at the cell surface (*11*) (Fig. 1B). In transiently transfected mammalian cells, T-WBP1 was not detected at the cell surface of unpermeabilized cells (Fig. 1C) and appeared to be localized in the ER in permeabilized cells (Fig. 1E). Pulse-chase analysis confirmed that T-WBP1 was resident in the ER because no carbohydrate processing was detected during a 6-hour period (Fig. 1F). As observed for similar mammalian ER retention motifs (*5*, *6*), a mutant of T-WBP1 in which both lysine residues were changed to serine (T-WBP1-SS) (Fig. 1A) was transported normally to the cell surface (Fig. 1, D and F). Mutants with the two lysine residues changed to arginine (T-WBP1-RR) or with four serine residues added at the COOH-terminus (T-WBP1+4S) (Fig. 1A) also lost ER retention and were transported to the cell surface (*12*). Thus, the yeast WBP1 di-lysine ER retention motif is functional in mammalian cells, which suggests conservation of the ER retention machinery between yeast and mammals.

To identify putative cytoplasmic receptors for the di-lysine ER retention motif, we constructed a chimeric protein containing the cytoplasmic domain of WBP1 fused to the COOH-terminus of glutathione-S-transferase (GST-WBP1). The fusion protein was expressed in bacteria and purified on glutathione-Sepharose beads. When yeast lysates were applied to the GST-WBP1 beads, we observed the specific binding of a 170-kD protein and of a set of proteins around 100 to 110 kD (Fig. 2A). These proteins did not bind to GST-

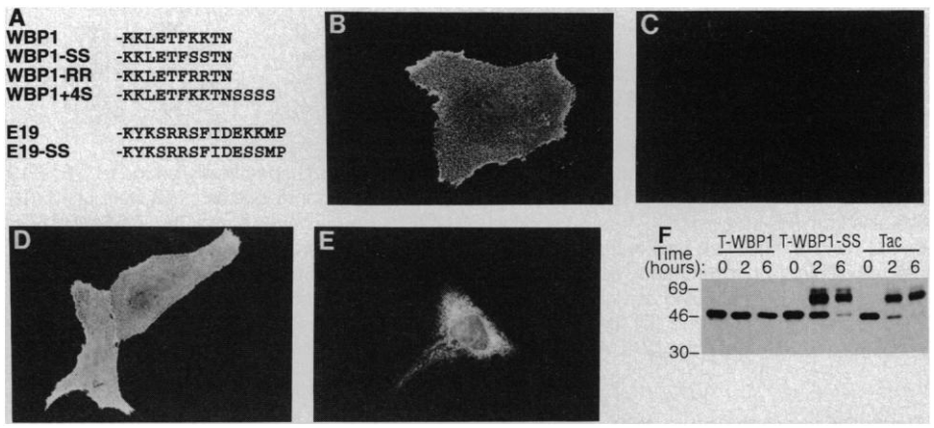


**Fig. 1.** Retention of Tac chimeras in the ER. (**A**) Sequences of the COOH-terminal domains of Tac and GST chimeric proteins (*2*, *23*). (**B** through **E**) Intracellular localization of Tac chimeras by immunofluorescence microscopy. Intact CV cells transfected with Tac (B), T-WBP1 (C), or T-WBP1-SS (D) were stained with an antibody to Tac and a rhodamine-conjugated donkey antiserum to mouse immunoglobin G. In (E), CV-1 cells transfected with T-WBP1 were fixed and permeabilized before the immunofluorescence staining. (**F**) Pulse-chase analysis of COS-1 cells transfected with Tac chimeras. COS-1 cells transfected with the indicated Tac chimeras were labeled for 15 min with [$^{35}$S]methionine and were then incubated for 0, 2, or 6 hours in the presence of unlabeled methionine. Sedimented cells were extracted with Triton X-100, and Tac proteins were isolated by immunoprecipitation before analysis by SDS-PAGE (10% gel) (*21*). The 7G7 antibody is directed against an epitope localized to the extracellular domain of the human Tac antigen (*24*). Protein standards are indicated at the left in kilodaltons. Pulse-chase and immunofluorescence experiments were done with CV-1 and COS-1 cells and gave identical results.

Basel Institute for Immunology, Grenzacherstrasse 487, Postfach CH-4005 Basel, Switzerland.

*To whom correspondence should be addressed.

WBP1-SS beads (Fig. 2A). When lysates from COS-1 cells were used, we observed the specific binding of a 170-kD protein and one protein of 100 kD (Fig. 2A). A specific band at 35 kD was also apparent on two-dimensional gels (*12*). We did not observe specific binding to GST-WBP1-SS, GST-WBP1-RR, or GST-WBP1+4S beads (Fig. 2B), which confirms that the proteins identified above can bind only to functional ER retention motifs. The absence of binding to GST-WBP1-RR and GST-WBP1+4S beads also ruled out the possibility that the binding of proteins to GST-WBP1 beads resulted from nonspecific interaction with the charged di-lysine motif. To verify that other di-lysine retention motifs could specifically bind the same proteins, we constructed a GST fusion protein containing the mammalian ER retention motif from the adenoviral E3/19K protein (GST-E19) (*5*) (Fig. 1A). The same set of proteins specifically bound to the GST-E19 beads but not to GST-E19-SS beads (Fig. 2C). To investigate why more proteins specifically bound to GST-WBP1 when yeast lysates were used, we lysed COS-1 cells in a buffer of lower ionic strength (Hepes-Triton). A pattern more similar to that observed with yeast lysates was then obtained, with at least two bands of 100 and 110 kD (Fig. 2D). Specific binding of a 61-kD protein could also be discerned (Fig. 2D). Nonspecific binding was also much higher under these conditions.

The molecular sizes of the proteins that bound to ER retention motifs are similar to those of the proteins that form the coatomer (*13*), a polypeptide complex involved in membrane traffic at the Golgi apparatus (*14*). We therefore did protein immunoblotting with antibodies to various mammalian coatomer subunits (COPs). The proteins from COS-1 cell lysates that bound to GST-WBP1 in Hepes-Triton buffer were recognized by antibodies to α-COP (170 kD), β-COP (110 kD), β′-COP (102 kD) (*15*), and γ-COP (98 kD) (Fig. 3A). No reactivity was observed with proteins adsorbed to GST-WBP1-SS beads (Fig. 3A). Identical results were obtained with two different monoclonal antibodies to β-COP, three different antisera to peptides from β′-COP, and two different antisera to peptides from γ-COP (*12*). The 61- and 35-kD proteins could correspond to δ- and ε-COPs, respectively, but antibodies to these COPs are not available (*13*). The α-COP could be completely removed from COS-1 cell lysates by incubation with GST-WBP1, and the apparent dissociation constant ($K_d$) was roughly estimated to be 1 nM (*12*). The 170-kD protein from yeast cell lysates that bound to GST-WBP1 was also recognized by the antibody to α-COP (*12*). When the cells were lysed in tris-Triton buffer before adsorption on GST-WBP1 beads, bound proteins showed strong reactivity with antibodies to α- and β′-COP but not with antisera to β- or γ-COP (Fig. 3B). This result suggests that partial coatomer complexes composed of α- and β′-COP and a 35-kD protein (probably ε-COP) also bind di-lysine ER retention motifs in tris-Triton buffer.

Our results demonstrate that di-lysine ER retention motifs interact specifically with the coatomer complex in vitro. Mutations that affect the function of the motif also abolish binding of the coatomer. Although we cannot assign binding to one individual subunit, the observation that partial complexes can bind in tris-Triton buffer restricts the search to the α-, β′-, or ε-COP. Alternatively, other proteins, undetected in our experiments, could serve as adapters between the coatomer and di-lysine motifs. The coatomer was originally purified from Golgi-derived coated vesicles (*13*) and is required for the budding of vesicles from Golgi membranes (*16*). It is still debated whether the coatomer plays a role in intra-Golgi, ER-to-Golgi, or Golgi-to-ER transport (*17, 18*). Our results suggest a possible role for the coatomer in retrograde Golgi-to-ER transport. According to this hypothesis, in post-ER compartments the coatomer would sort di-lysine–tagged proteins to vesicles destined to the ER, thus preventing further transport of those proteins along the exocytic pathway. This hypothesis is reinforced by recent observations suggesting that ER retention mediated by di-lysine motifs is achieved by continuous retrieval to the ER of tagged proteins from post-ER compartments (*8*). Genetic and biochemical approaches will be necessary to further evaluate the physiological role of the coatomer in retrograde Golgi-to-ER transport and ER retention.

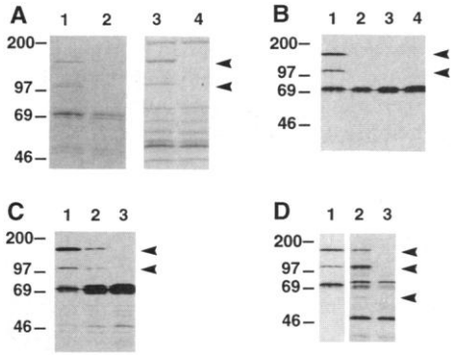


**Fig. 2.** Interaction of yeast and mammalian proteins with fusion proteins containing di-lysine motifs. (**A**) Coomassie blue staining of yeast (lanes 1 and 2) and COS-1 (lanes 3 and 4) proteins bound to GST-WBP1 (lanes 1 and 3) or GST-WBP1-SS (lanes 2 and 4) fusion proteins immobilized on glutathione-Sepharose beads (*25*). (**B**) Requirement of functional ER retention motifs for specific protein binding. COS-1 cells ($10^6$) were metabolically labeled overnight with [$^{35}$S]methionine and [$^{35}$S]cysteine and solubilized in tris-Triton buffer (*25*). Lysates were incubated with beads coated with GST-WBP1 (lane 1), GST-WBP1-SS (lane 2), GST-WBP1+4S (lane 3), and GST-WBP1-RR (lane 4), and beads were processed as in (A). (**C**) The same set of proteins interacting with GST-WBP1 beads (lane 1) specifically bound to GST-E19 beads (lane 2) but not to GST-E19-SS beads (lane 3). The same conditions as in (B) were used. (**D**) Proteins from $^{35}$S-labeled COS-1 cells bound to GST-WBP1 beads (lanes 1 and 2) or GST-WBP1-SS beads (lane 3) after cell lysis in tris-Triton buffer (lane 1) or in Hepes-Triton buffer (lanes 2 and 3) (*25*). In all binding experiments, equivalent amounts of GST fusion proteins were used, as assayed by Coomassie blue staining of eluted GST products. Protein standards are indicated at the left in kilodaltons. In (B), (C), and (D), proteins were separated by SDS-PAGE (8% gels) and gels were processed for fluorography (*21*). Arrowheads indicate specifically bound proteins.

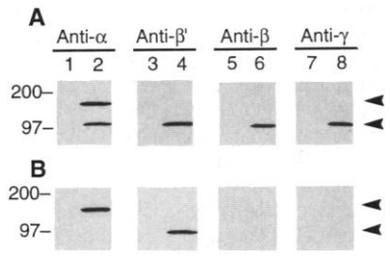


**Fig. 3.** Coatomer binding to WBP1 di-lysine motif. (**A**) Proteins adsorbed to GST-WBP1-SS beads (lanes 1, 3, 5, and 7) or GST-WBP1 beads (lanes 2, 4, 6, and 8) in Hepes-Triton buffer were identified by protein immunoblotting with an antibody to α-COP (lanes 1 and 2), β′-COP (lanes 3 and 4), β-COP (lanes 5 and 6), or γ-COP (lanes 7 and 8) (*26*). In addition to the 170-kD α-COP protein, the antibody to α-COP reacted with a 100-kD band that could correspond either to a cross-reactive protein or to an α-COP degradation product. (**B**) Binding of a partial coatomer complex to GST-WBP1 beads in tris-Triton buffer. The same experiment as in (A) was repeated, but proteins were adsorbed in tris-Triton buffer. Protein standards are indicated at the left in kilodaltons. Arrowheads indicate specifically immunodetected proteins.

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26. Binding of COS-1 cell lysates ($10^{6}$ cells per lane) to GST beads was done as in Fig. 2. After SDS-PAGE (8% gels), proteins were transferred to nitrocellulose membranes. Membranes were blocked with PBS containing low fat milk (5%) and Tween-20 (0.1%) for 1 hour and then incubated with specific antibodies. Proteins were visualized with an Enhanced Chemiluminescence kit (Amersham) according to the manufacturer's instructions.
27. The rabbit antiserum to an $NH_2$-terminal peptide from α-COP, the rabbit antiserum to an $NH_2$-terminal peptide from β′-COP (771), and the rabbit antiserum to γ-COP (800) were provided by C. Harter and F. T. Wieland. The monoclonal antibody to a peptide from β-COP (MaD) (*18*) came from T. E. Kreis. We thank C. Harter, F. T. Wieland, and T. E. Kreis for antibodies; H. Riezman and R. D. Klausner for helpful discussions; S. Even and S. Hennecke for excellent technical assistance; and I. Chretien, G. M. Griffiths, J. A. Garcia-Sanz, and J. Kaufman for critical review of this manuscript. The Basel Institute for Immunology was founded and is supported by F. Hoffmann–La Roche, CH-4005 Basel, Switzerland.


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