Molecular Basis for Recognition of Dilysine Trafficking Motifs by COPI

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INTRODUCTION

In addition to its essential role in membrane trafficking (Duden et al., 2003), COPI has been implicated in lipid homeostasis (Beller et al., 2008), viral replication (Cureton et al., 2012), and pathogen entry (Guo et al., 2008; Misselwitz et al., 2011). COPI facilitates retrieval of endoplasmic reticulum (ER) resident proteins from the Golgi to the ER, cycling of proteins between the ER and Golgi, and retrograde transport of proteins within the Golgi stack (Popoff et al., 2011). Many type I transmembrane proteins transported by COPI bear C-terminal dilysine-based motifs (Jackson et al., 1990; Letourneur et al., 1994; Townsley and Pelham, 1994). Lysines are strictly required at the -3 and -4 (KKxx) or -3 and -5 (KxKxx) positions relative to the C terminus and cannot be replaced by histidines or arginines (Jackson et al., 1990; Schröder-Köhne et al., 1998). Transplanting KKxx (Nilsson et al., 1989) or KxKxx motifs (Jackson et al., 1990) onto CD8 reporters results in ER retention, whereas removal of dilysine motifs from ER resident proteins results in cell surface expression (Pääbo et al., 1986). Proteins with dilysine motifs include yeast Wbp1 (Gaynor et al., 1994) and mammalian OST48 (Silberstein et al., 1992), which are essential components of N-linked oligosaccharyl transferase complexes in the ER, and yeast Emp47 (Schröder et al., 1995) and its mammalian ortholog, ERGIC-53, which cycle luminal glycoproteins between the ER and Golgi (Schröder et al., 1995; Teasdale and Jackson, 1996; Schindler et al., 1993). Certain viral glycoproteins also contain dilysine motifs (Pääbo et al., 1986; Goepfert et al., 1997), allowing viruses to circumvent host immune defenses (Pääbo et al., 1987).

The heptameric COPI complex (α/β/β'/γ/δ/ε/ζ) is recruited on block onto Golgi membranes (Hara-Kuge et al., 1994) but can be divided conceptually into two subcomplexes. The β/β'/γ/ζ F-subcomplex is structurally and functionally similar to AP clathrin adaptor complexes (Yu et al., 2012). The α/β/ε B-subcomplex has been likened to clathrin because α-COP, β-COP, and clathrin contain N-terminal WD-repeat (also referred to as β-propeller) domains and C-terminal α-solenoid domains and because recent studies have suggested that β-COP, like clathrin, can form trimers (Lee and Goldberg, 2010). Whereas β/β'/γ/ζ and the AP complexes evolved from a common ancestor (Schledzewski et al., 1999), α- and β'-COP evolved independently of clathrin (Schledzewski et al., 1999). Very little is known about the molecular mechanisms of cargo recognition in the COPI-mediated retrograde pathway as compared to post-Golgi clathrin-mediated pathways. Controversy has long existed in the literature regarding the basis of dilysine cargo binding. Initial studies indicated that dilysine motifs bind the F-subcomplex (Harter et al., 1996). Other reports implicated components of the B-subcomplex (Cosson and Letourneur, 1994; Lowe and Kreis, 1995; Fiedler et al., 1996; Schröder-Köhne et al., 1998; Eugster et al., 2004; Béthune et al., 2006). Subsequent yeast genetic and two hybrid evidence led to the proposal that N-terminal WD-repeat domains of α- and β'-COP were responsible for differential binding to KKxx and KxKxx motifs, respectively (Eugster et al., 2004; Schröder-Köhne et al., 1998). Defects in KKxx cargo trafficking were observed in temperature sensitive yeast strains harboring mutations that cause protein misfolding (data not shown) in the α-COP N-terminal WD-repeat domain (Letourneur et al., 1994; Schröder-Köhne et al., 1998) or in strains lacking the domain altogether (Eugster et al., 2000). Similarly,
deletion of the β'-COP N-terminal WD-repeat domain resulted in missorting of KxKxx signals, and loss of both α- and β'-COP domains is lethal in yeast (Eugster et al., 2004). However, a recent X-ray crystal structure of an αβ'-COP subcomplex instead proposed that the β'-COP N-terminal WD-repeat domain undertakes a structural role by forming a trimer that constitutes the vertex of a COPI cage (Lee and Goldberg, 2010).

We demonstrate that N-terminal WD-repeat domains of α- and β'-COP directly bind dilysine motifs and establish the molecular bases for the interactions using X-ray crystallography, structure-directed mutagenesis, and isothermal titration calorimetry (ITC). Finally, we show that dilysine cargo trafficking is mediated through binding of the motifs to the N-terminal WD-repeat domains of α- and β'-COP in vivo.

**RESULTS**

**Structure of β'-COP 1-304 with a KxKxx Motif**

ITC experiments conclusively showed that dilysine motifs bound robustly with a low micromolar $K_D$ to the β'-COP N-terminal WD-repeat domain (see next section). Attempts to crystallize this domain were made in the presence of a variety of KKxx- and KxKxx-containing peptides based on endogenous motifs (Table S1 available online). The first structure obtained was that of yeast β'1-304His6 in the presence of the CTFKKTN motif derived from yeast Wbp1 (Table 1; Supplemental Experimental Procedures; Figures S1 D–S1G). This structure, solved at 1.8 Å resolution by SIRAS, showed that the WD-repeat domains pack one on top of the other in the lattice. The C-terminal His6 tag from one molecule reaches to the top surface of its neighbor, specifically occupying a charged surface patch (Figure S1 G). This structure suggested a potential binding site for short basic linear motifs, particularly highlighting the importance of a terminal carboxylate group and backbone carbonyl binding. The CTFKKTN peptide included in crystal trials was not visible in the density but was required for crystallization, likely forming weak interactions that facilitated crystal packing. As a result, we used an untagged β'1-304 construct in all further studies and subsequently obtained structures of β'1-304 in complex with CTFKKTKTN and CTFKKT Knoxville peptides. Because both structures reveal the same
mode of binding via the carboxy terminii and -3 lysines of motifs, we present here the structure of β'1-304 with its preferred ligand, CTFKTKTN.

All eight residues of the peptide are visible in the 1.8 Å resolution complex (Figures 1A–1C and S1A–S1C; Table 1). Interaction between β'1-304 and the KxKxx motif is mediated primarily by electrostatic contacts. The peptide carboxy terminus is located on a positively charged patch formed by guanidinium groups of R15 and R59 and the amino group of K17 in the center of the top surface of the WD-repeat domain. The -3 lysine interacts with a negatively charged patch formed from carboxylate groups of D206 and E248. In a negative patch formed by D98 and D117, a lysine residue (K261) from a symmetry copy (gray residues) occupies the negative patch formed by D98/D117 (view rotated 90°). The linker between β’-COP N- and C-terminal domains is flexible. β’1-604 (PDB ID code 3MKQ) is shown from gold to lemon (N to C terminus); β’1-604 with CTFKTKTN in this study is shown from dark to light green (N to C terminus) with peptide in yellow.

The final key structural feature is the location of the -2, -3, and -4 carbonyl groups of the motif, which project down into a basic groove.

The WD-Repeat Domains of β’-COP Exhibit Inherent Flexibility

A recently published crystal structure of β’-COP (Protein Data Bank [PDB] ID code: 3MKQ) suggested that its N-terminal WD-repeat domains mediate formation of a trimer (Lee and Goldberg, 2010). In contrast, we do not observe trimer formation of the N-terminal WD-repeat domain in any of our structures of either β’1-304 with different peptides (Supplemental Experimental Procedures) or β’1-604 with CTFKTKTN (Figures 1D and S2; Table 1). As in published work (Lee and Goldberg, 2010), we find no evidence of trimer formation in solution by multilateral light scattering (Supplemental Experimental Procedures), suggesting an approximate millimolar Kₐ. However, our β’1-604 structure demonstrates that there must be significant conformational flexibility between the two WD-repeat domains of β’-COP because the C-terminal domain undergoes a ~96° screw rotation (Figures 1D and S2) relative to 3MKQ, when the structures are superposed on their N-terminal domains. If the interface between the C-terminal WD-repeat domain and α-solenoid is rigid, rotation of the C-terminal domain by ~96° would rotate each α-solenoid domain by ~180° relative to 3MKQ, resulting in a trimer of the opposite hand (Figures 1D and S2).

β’- and α-COP N-Terminal WD-Repeat Domains Bind Dilysine Motifs In Vitro

Having proposed residues as important for binding of the β’-COP N-terminal WD-repeat domain to KxKxx motifs (Figures 1C, 2A, and 2B), we tested our model in solution using a combination of ITC and structure-based mutagenesis. For all...
biophysical experiments, the KxKxx motif from wild-type yeast Emp47 (RQEIKTKLL) was used instead of the synthetic peptide used in crystallization, demonstrating the disulfide bond observed in the structure is not required for binding in solution. Wild-type yeast β’-COP 1-304 binds the KTKLL motif with a $K_D$ of $6.8 \pm 2.6 \, \text{mM}$ (Figure 2C). β’-COP exhibits differential binding to KKxx and KxKxx signals in vitro: the affinity for the Wbp1 KKTN motif (Figure 2D) is weaker by an order of magnitude ($K_D/C_24 = 85 \, \text{mM}$), and hence we hypothesize that a lysine at the -4 position likely cannot interact optimally with the patch formed of D98 and D117 (Figure 2A). For two reasons, we cannot draw conclusions regarding preference of β’-COP for a -5 lysine over a -4 lysine from comparing structures of β’-COP 1-304 with different peptides. First, formation of the disulfide bond between the peptide cysteine and C220 “pulls” the N-terminal end of the peptide toward a symmetry copy in the crystal lattice, altering the backbone conformation. Second, K261 from a symmetry copy occupies the patch formed of D98 and D117 in both structures. These features cause lysines at either the -4 or -5 positions to relocate into the solvent channel, where they are not visible in unbiased electron density (Figure S1B). However, the importance of the D98/D117 patch for -5 lysine binding is confirmed by mutagenesis (below).

We next constructed mutants to confirm importance of specific residues in the motif-binding site. The β’-COP R15A K17A R59A mutant was designed to disrupt binding to the carboxy terminus of the motif, and the D206A E248A mutant was intended to disrupt binding to the -3 lysine. Based on indirect evidence from structures, we predicted a D98A D117A mutant would abolish interaction with the -5 lysine. This prediction was based on the position of a histidine residue equivalent to the -5 lysine in the β’-COP 1-304 structure (Figure 2C), both of which occupy the D98/D117 patch. Binding of all three mutants to RQEIIKTKLL was undetectable by ITC ($K_D < 300 \, \text{mM}$) (Figure 2C), without affecting overall protein fold as judged by circular dichroism (CD) and gel filtration elution profiles (data not shown).

We also tested the effect of altering key determinants in the KxKxx motif (Figure 2C). Wild-type β’-COP 1-304 exhibits no measurable binding to an amidated peptide (KTKLL-CONH$_2$) or to KTSLL and weak binding to STKLL ($K_D \sim 160 \, \text{mM}$).
Together with our structures, these data indicate the key motif determinants are the -3 lysine and carboxy terminus, which are present in both KxKxx and KKxx motifs. In addition to the carboxylate group binding to the basic patch formed of R15/K17/R59, two carbonyl groups from the -2 and -3 positions in the motif backbone must fit into a groove formed by R59 and R101; the carbonyl group at the -4 position also likely contacts R101, as observed in the \( \beta' \)-COP structure (Figure 2B). Fulfilling these two requirements restricts the placement of a lysine residue such that it can only be accommodated in the patch formed by D206 and E248 when located two residues away from the carboxy terminus (i.e., at the -3 position). Testing of potential variant basic motifs further confirmed these results: the KKLIE peptide from p23, which lacks a -3 lysine, does not bind \( \alpha \)-COP (Figure 2D). In agreement with previous work (Fiedler et al., 1996), arginines cannot replace lysines: we could detect no binding to an RRVV peptide from p24 (Figure 2D). When carbonyl groups in the peptide backbone sit in the basic groove, an arginine side chain is too long to be accommodated at the -3 position.

\( \alpha \) and \( \beta' \)-COP arose from a single gene by duplication and thus possess conserved domain architecture. The N-terminal WD-repeat domains exhibit 51% similarity and 18% identity at the amino acid level (data not shown), and hence we should be able to reliably model the \( \alpha \)-COP structure. Because all of the residues in the \( \beta' \)-COP dilysine motif binding site are conserved in \( \alpha \)-COP (Figure 3A), the \( \alpha \)-COP domain must possess analogous charged patches on its top surface (Figure S3C), pointing to a conserved mechanism of motif binding. Using structures of \( \beta' \)-COP/KxKxx motif complexes, we generated a homology model of residues 1–327 of yeast \( \alpha \)-COP and modeled the interaction with the Wbp1 KKTN motif (Figure S3). The three common motif determinants (carboxy terminus, backbone carbonyl groups, and -3 lysine) should bind in the same way, with the only difference being the preference for a lysine at the -4 position.

As predicted, analogous mutations to those proposed for \( \beta' \)-COP introduced into yeast \( \alpha \)-COP gave comparable results for KKTN motif binding as determined by ITC, without affecting protein fold (CD and gel filtration profiles; data not shown). R15A K17S R59S disrupted binding to the motif carboxy terminus, and D229A E273A disrupted binding to the -3 lysine (Figure S3B). Although we have no experimental evidence for \( \alpha \)-COP and indirect evidence from \( \beta' \)-COP structures, we attempted to model how the -4 lysine binds on the \( \alpha \)-COP surface. By analogy with \( \beta' \)-COP, we hypothesized that D96 and D115 comprise the binding site for the -4 lysine and designed a D96A D115A mutant. These two residues are absolutely conserved between both \( \alpha \)- and \( \beta' \)-COP from yeast to humans (Figure 3A). In addition, the short motif would be able physically to bridge the distance between D96/D115 and the other two patches known to be important for binding. The closest additional negative patch on the surface of our model (D296/D315) is located too far away (~13Å) from the C-\( \alpha \) of the -3 lysine to be involved. As predicted, the D96A D115A mutant abolished binding to KKxx motifs in vitro (Figure S3B).
**α- and β'-COP Traffic Dilysine-Based Reporter Constructs in Yeast**

Finally, we tested our structural model of dilysine motif binding by COP1 in *Saccharomyces cerevisiae*. Using homologous recombination, we generated strains by replacing endogenous SEC27 (β'-COP) or RET1 (α-COP) genes with structure-based point mutants described above and then introduced dilysine reporter constructs. We then observed how wild-type-replaced and point-mutant-replaced strains transported dilysine cargo. Based on published information (Gaynor et al., 1994; Letourneau et al., 1994; Schröder-Köhne et al., 1998), we predicted wild-type-replaced strains would recycle reporters back to the ER, whereas mutant strains would fail to recycle reporters, leading to vacuolar degradation. Results indicated that our structural model correctly describes interaction of the β'-COP N-terminal WD-repeat domain with dilysine motifs. All mutations in SEC27 that disrupt binding to the KxKxx motif resulted in significantly lower steady-state levels of the KxKxx reporter. Reporter levels in all mutant strains are 30%–40% of wild-type levels prior to the cycloheximide chase (Figure 3B). One hour after the chase, the majority of the reporter (~90%–95%) has been degraded in the vacuole. In contrast, the wild-type-replaced strain maintained similar levels of the reporter over the time course of the experiment. A Kkxx reporter (Figure S4A) also exhibited lower steady-state levels and degradation in the vacuole. These phenotypes agree with structural and in vitro binding results, confirming that β'-COP can bind both types of motif (Figure 2D).

Likewise, our α-COP homology model correctly predicted critical residues for binding the carboxy terminus (R13A K15S R57S mutant) and -3 lysine (D229A E273A mutant) of Kkxx motifs. Disrupting these patches in RET1 resulted in steady-state Kkxx reporter levels of ~40% and loss of ~80% of the reporter over time, compared to wild-type levels (Figure 3B). The effect is significant but less pronounced than effects on degradation of the Kkxx reporter in sec27::ura3 mutant strains. Both in vitro and in vivo data suggest that β'-COP can bind to and thus transport Kkxx motif-containing cargo (endogenous and reporter-based) in the absence of a functional α-COP motif binding site. Because the Kkxx motif is more prevalent in yeast, our results together suggest that β'-COP carries the bulk of reporter in our strains.

We observed a less significant steady-state effect (~85% of wild-type levels) and less degradation (loss of ~65%) in the ret1 D96A D115A mutant predicted to bind the -4 lysine, suggesting this mutant still shows functionally significant binding to Kkxx motifs. This reflects uncertainty inherent in the α-COP homology model. We do not know the absolute position and orientation of the α-COP determinant residues located on the conserved patch, and other contacts may be involved in -4 lysine binding. Indeed, the charge and shape of the D229/E273 patch may be able to accommodate both the -3 and -4 lysines. An X-ray structure of the α-COP N-terminal WD-repeat domain with Kkxx motif will be required to understand the -4 lysine binding preference. Thus far, the α-COP N-terminal domain from multiple organisms (yeast, fly, and humans) has proven refractory to crystallization despite extensive efforts (Supplemental Experimental Procedures).

One possible explanation for the observed phenotypes would be that our point mutations have disrupted formation and thus all functions of COPI. However, in addition to confirming that point mutations did not disrupt folding of recombinant N-terminal WD-repeat domains, three lines of evidence suggested that the COPI coat was intact and functional in mutant strains (Figure S4). First, COPI subunits (F-/B-subcomplexes) were expressed at similar levels in mutant and wild-type replacement strains (Figure S4C), demonstrating that point mutations have not disrupted complex formation. Second, none of the mutant strains exhibited temperature sensitivity up to 37°C (Figure S4D), even though both SEC27 and RET1 are essential genes. Finally, in point-mutant-replaced strains, the syntaxin Sed5, which cycles between the ER and Golgi (Wooding and Pelham, 1998; Weinberger et al., 2005), is present at levels found in wild-type cells (Figures 3B, S4A, and S4B), indicating that COPI is functional for nondilysine-based retrograde transport from the Golgi.

Finally, we generated a yeast strain in which both the α- and β'-COP N-terminal WD-repeat domains had lost the ability to bind the carboxy terminus of dilysine motifs (sec27::ura3 R15A K17A R59A ret1::trp1 R13A K15S R57S). Although viable at 37°C, the ability of this mutant to traffic both Kkxx and Kkxx reporter constructs was severely impaired (Figure S4E). The inability to support retrograde dilysine-based transport causes a slight growth defect at 37°C, resulting in smaller colonies as compared to wild-type, but this mutant is not lethal.

**DISCUSSION**

A molecular explanation of cargo binding by the COPI coat complex has long proven elusive. We have demonstrated here how the N-terminal WD-repeat domains of COPI B-subcomplexes recognize the most commonly used retrograde trafficking signals, dilysine-based motifs. Unlike previously used temperature-sensitive and deletion strains that result in general protein misfolding, our highly specific tools selectively abolish a single function of coatomer and will open the way for in vivo investigation of retrograde trafficking, including roles and importance of dilysine-based cargo in COPI coat recruitment and other cellular functions.

COPI coat assembly requires both membrane and cargo binding (Hara-Kuge et al., 1994; Lowe and Kreis, 1995; Bremer et al., 1999). To bind their cargo, α- and β'-COP N-terminal WD-repeat domains must approach very close to the membrane (~20 Å) because the minimum spacing between a transmembrane domain and C-terminal dilysine motif found in endogenous cargo is five amino acid residues. In addition to binding cargo, the β'-COP N-terminal WD-repeat domain has been proposed to form a trimer (Lee and Goldberg, 2010), and cryoelectron tomography data from reconstituted COPI-coated vesicles demonstrate that a portion of density with 3-fold local symmetry is located proximal to the membrane (Faini et al., 2012). It is tempting to speculate that this density corresponds to trimers of β'-COP (and possibly α-COP) N-terminal WD-repeat domains. Indeed, a model of Kkxx motif binding to trimeric β'-COP, the mechanism of which we have described here, indicates there is sufficient space within the proposed interface for each WD-repeat domain to bind a single Kkxx motif (Figure S2B). It is therefore possible that β'-COP N-terminal WD-repeat domains simultaneously bind cargo and mediate trimerization. However, fitting of the whole 3MKQ trimeric structure (Lee and Goldberg, 2010) would be that our point mutations have disrupted formation and thus all functions of COPI. However, in addition to confirming that point mutations did not disrupt folding of recombinant N-terminal WD-repeat domains, three lines of evidence suggested that the COPI coat was intact and functional in mutant strains (Figure S4). First, COPI subunits (F-/B-subcomplexes) were expressed at similar levels in mutant and wild-type replacement strains (Figure S4C), demonstrating that point mutations have not disrupted complex formation. Second, none of the mutant strains exhibited temperature sensitivity up to 37°C (Figure S4D), even though both SEC27 and RET1 are essential genes. Finally, in point-mutant-replaced strains, the syntaxin Sed5, which cycles between the ER and Golgi (Wooding and Pelham, 1998; Weinberger et al., 2005), is present at levels found in wild-type cells (Figures 3B, S4A, and S4B), indicating that COPI is functional for nondilysine-based retrograde transport from the Golgi.

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**Experimental Procedures**

For constructs, antibodies, and yeast strains used in this study, see the Supplemental Experimental Procedures.
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