Peptide-in-groove interactions link target proteins to the β-propeller of clathrin

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The "WD40" domain is a widespread recognition module for linking partner proteins in intracellular networks of signaling and sorting. The clathrin amino-terminal domain, which directs incorporation of cargo into coated pits, is a β-propeller closely related in structure to WD40 modules. The crystallographically determined structures of complexes of the clathrin-terminal domain with peptides derived from two different cargo adaptors, β-arrestin 2 and the β-subunit of the AP-3 complex, reveal strikingly similar peptide-in-groove interactions. The two peptides in our structures contain related, five-residue motifs, which form the core of their contact with clathrin. A number of other proteins involved in endocytosis have similar "clathrin-box" motifs, and it therefore is likely that they all bind the terminal domain in the same way. We propose that a peptide-in-groove interaction is an important general mode by which β-propellers recognize specific target proteins.

Characteristic "interaction domains," of various conserved designs, often are responsible for specific protein associations in sorting and signaling pathways. One example is the β-propeller formed by seven "WD40 repeats"—modules, such as those in heterotrimeric G proteins, that contain about 40 residues with a tryptophan (W) and an aspartic acid (D) at defined positions (1–3). Each repeat forms a four-strand β-sheet—one blade of the propeller-like domain. The "terminal domain" of clathrin has an essentially identical propeller structure, although it lacks tryptophans and aspartic acids at the canonical positions in most of its seven blades (4).

Clathrin is a three-legged ("triskelion"-shaped) molecule (5, 6) that forms cage-like enclosures (7–9) and drives vesiculation of cellular membranes (10–13). Each clathrin leg is an $\approx$1,600-aa heavy chain, with the $\approx$330-residue, amino-terminal β-propeller domain at its tip (14–16). The terminal domain connects to a set of about 42 α-zigzags, which forms the 450-Å-long extended part of the leg and which ends in a small trimerization domain at the carboxyl terminus (4, 17). The assembling clathrin lattice recruits membrane-anchored cargo to the coat through adaptor proteins, which interact with the terminal domain (18–21). One particular adaptor, β-arrestin, directs internalization of β_2-adrenergic receptors through the clathrin-endocytic pathway (22–24). β-Arrestin attaches to clathrin through a short peptide near its carboxyl terminus (25). The crystal structure of α-arrestin, the corresponding protein in the visual system, shows that the clathrin-interacting segment in nonvisual arrestins would be part of a carboxyl-terminal flexible loop (26). Clathrin residues that influence binding with this extended element from β-arrestins lie in the groove between blades 1 and 2 of the terminal domain (4, 25).

The structures reported here show that the interacting segment of β-arrestin runs along the groove and also demonstrate that β-subunits of the heterotetrameric clathrin adaptors interact with the same site. We suggest further that other seven-blade propellers, such as those containing consensus WD40 repeats, recognize some of their interaction targets through similar peptide-in-groove interactions.

Materials and Methods

Expression and Purification of td40. To express the clathrin td40 fragment (residues 1–363), we used the vector pBAT4 in *Escherichia coli* strain BL21(DE3), grown at 37°C. After 4 hr of induction with 0.1 mM isopropyl β-D-thiogalactoside, cells were lysed by sonication in 20 mM Tris (pH 8.0)/50 mM NaCl/1 mM EDTA (buffer A), and the lysate was fractionated on a HPQ-Sepharose column in buffer A with a 50- to 500-mM NaCl gradient. The td40 fragment eluted at 200 mM NaCl. Pooled fractions were mixed with ammonium sulfate to a final concentration of 1.6 M; the solution was centrifuged at 33,000 $\times$ g for 10 min at 4°C, and the supernatant was loaded onto a 7-ml phenyl-Sepharose column and eluted with a 60–0% ammonium sulfate gradient in buffer A. The fractions containing the terminal domain were collected, concentrated, and loaded on a Superdex 75 column for size-exclusion chromatography in buffer A. The td40 eluted as a monomeric species, which was concentrated to about 18 mg/ml ($\approx$0.4 mM).

Crystallization of Peptide–td40 Complexes. The peptides containing the clathrin-binding sites of β-arrestin 2 (sequence: VDTNLIEFETNYA), of β1- and β2-hinge (sequence: GDLLNLDLGP), and of the β3a-hinge (sequence: KDVSLLDLDDFN) were synthesized by using standard fluorenylmethoxycarbonyl chemistry. The β-arrestin 2 peptide was dissolved in 1 M Tris (pH 8.0) at a concentration of 15 mM; the β2- and β3-hinge peptides were dissolved in 100 mM Tris (pH 8.0) at a concentration of 10 mM. The peptides were added to td40 in 2- to 4-fold molar excess. The protein–peptide complexes crystallized in hanging drops after 4 days (at 4°C for the β-arrestin 2–td40 and at room temperature for the β2- or β3-hinge–td40 complexes). The equilibration solution was 21% polyethylene glycol (PEG) 4000/350 mM potassium acetate/1 mM DTT/100 mM Tris, pH 8.0. The crystals with the β-arrestin 2 peptide were transferred to 25% PEG 4000/350 mM potassium acetate/1 mM DTT/100 mM Tris, pH 8.0, soaked for 2 hr, dialyzed overnight against 25% PEG 4000/350 mM potassium acetate/30% glycerol/100 mM Tris, pH 8.0, flash-frozen, and stored in liquid nitrogen. The crystals with peptides from the β2- or β3-hinge were soaked for 40 min at each step and then flash-frozen. Both complexes crystallize in space group C2 with two complexes per asymmetric unit. The cell dimensions for the crystals of td40 with the β-arrestin 2 peptide are a = 137.6, b = 130.8, c = 78.5, and β = 115.5; those for td40 with the β3-hinge peptide are a = 137.0, b = 131.3, c = 79.0, and β = 116.3.

Structure Determinations. Diffraction data were collected at −160°C in 1° oscillation frames by using an 18- or a 30-cm MAR image plate scanner with an Elliott GX13 rotating anode source. Intensities were integrated and scaled with the HKL suite of programs (27). The overall R_{merge} was 4.3% to 2.9 Å for the

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 1C9L and 1C9I).

See commentary on page 960.

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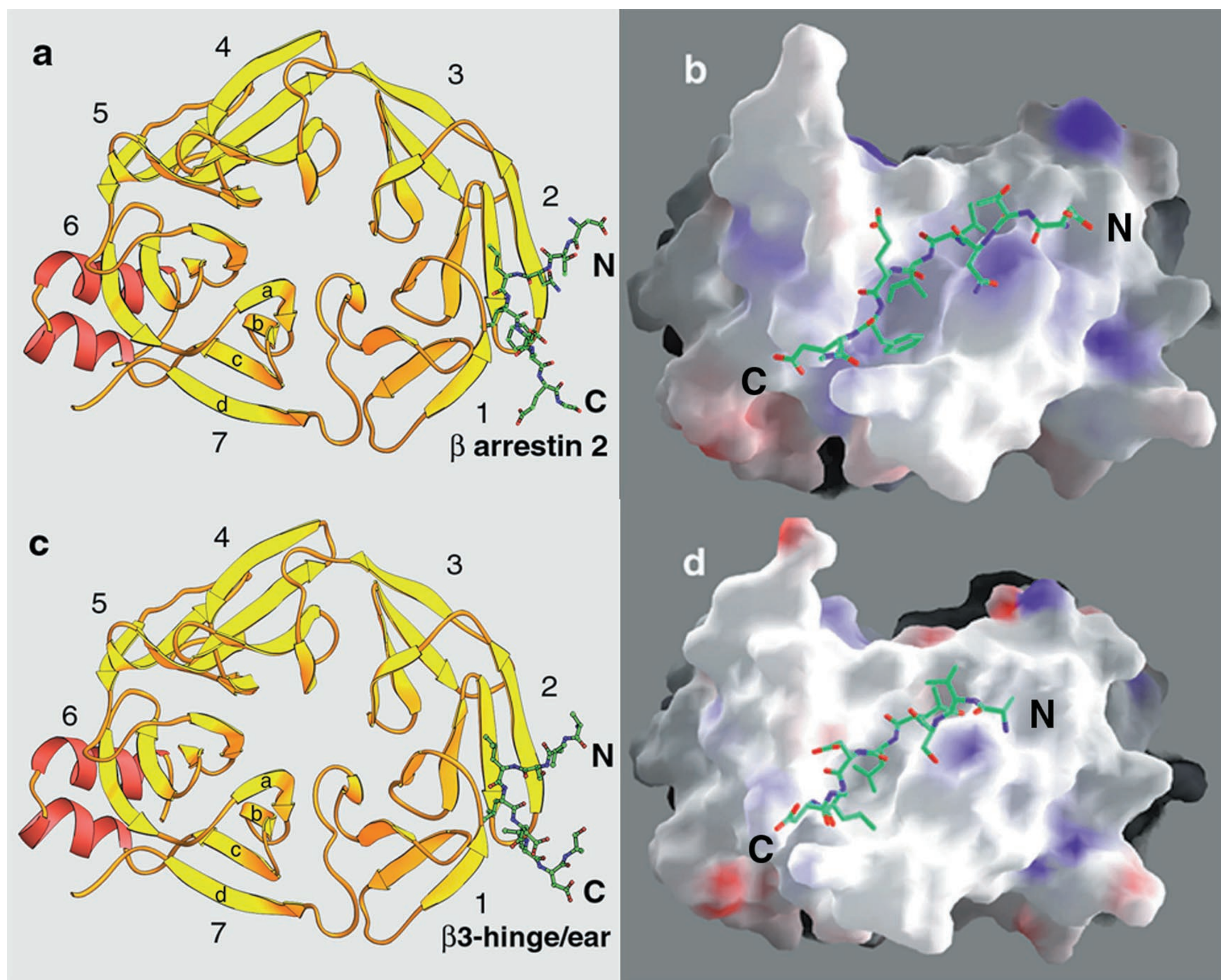

Fig. 1. Structures of clathrin-terminal domain complexed with clathrin-box peptides from β-arrestin 2 (*a* and *b*) and β3-hinge of AP-3 (*c* and *d*). (*a* and *c*) Ribbon diagrams of the complexes. The representation is a ''top'' view of the td40 fragment (residues 3–359) looking from the membrane toward the surrounding clathrin coat. The propeller blades are numbered 1–7; each blade contains four antiparallel β-strands, labeled a–d. Ordered portions of the bound peptides from β-arrestin 2 and β3-hinge (of sequence DTNLIEFE and VSLLDLD) are in green. The figure was made with RIBBONS (31). (*b* and *d*) Surface representation of the complexes. The images of *b* and *d* have been rotated approximately 90° toward the viewer. The map shows positive (blue), negative (red), and hydrophobic/neutral patches (white) projected onto the surface representation. The figure was made with GRASP (32).

β-arrestin 2 peptide/td40 complex and 9.6% to 2.7 Å resolution for the AP-3 peptide/td40 complex. A molecular replacement solution was obtained by using the program AMORE (28) in its CCP4 version (29) with the clathrin-terminal domain (1bpo), truncated to residue 363, as the search model. Refinement was carried out by using CNS (30) with noncrystallographic symmetry restraints. After initial rigid-body refinement, residues in close proximity to the electron density of the peptides were truncated to alanines to avoid local bias, and the protein models were subjected to two cycles of torsion-angle dynamics before fitting a polyalanine model into the peptide density. Subsequent cycles of torsion-angle and grouped B factor refinement, side-chain fitting (for the peptide and its contacts), and some rebuilding led to models containing the following residues: for the β-arrestin 2 complex, 3–359 for the two clathrins in the asymmetric unit and 370–377 for the peptide; and for the β3-hinge complex, 3–357 and 4–358 for the two clathrins and 815–823 and 816–822, respectively, for the peptide. These models were subjected to a final round of torsion angle dynamics and grouped B factor, individual B factor, and positional refinement. The R and R_{free} were 22.0 and 26.3, respectively, for the β-arrestin 2/td40 complex; they were 22.7 and 26.8 for the β3-hinge/ear/td40 complex. The coordinates have been deposited in the Protein Data Bank (ID codes 1C9L and 1C9I).

Results and Discussion

The clathrin-terminal domain/linker fragment we crystallized previously contains 5 α-zigzags in addition to the amino-terminal β-propeller module (4). The first two helices are tightly packed against a nonpolar surface of the propeller. Because it is likely that they fold in concert with the propeller, we expressed a 40-kDa fragment, termed td40, that includes this pair of helices as well as the entire propeller region (residues 1–363) (Fig. 1 *a* and *b*). The key interacting residues in the peptide from β-arrestin 2 resemble sequences in other adaptor proteins, such as a segment in the ''hinge'' of the β-subunits of the heterotetrameric adaptor complexes AP-1, AP-2, and AP-3 (Fig. 2). We therefore also determined the crystal structure of the clathrin-terminal domain with a

CLATHRIN - BOX
L L p L(-)

Protein	Position	Preceding	Clathrin box	Following
AP-1 β 1	628	L L G D	**L L N L D**	L G P P
AP-2 β 2	627	L L G D	**L L N L D**	L G P P
AP-3 β 3a	814	K D V S	**L L D L D**	D F N P
β - arrestin 1	373	V D T N	**L I E L D**	T N D D
β - arrestin 2	369	V D T N	**L I E F E**	T N Y A
amphiphysin 1	347	K E E T	**L L D L D**	F D P F
amphiphysin 2	386	E Q A S	**L L D L D**	F D P L
AP180	174	Q I D A	**L L E F D**	V H P N
LAP	188	Q L D A	**L L E F D**	C Q S N
epsin1	452	P N A A	**L V D L D**	S L V S

Fig. 2. Alignment of sequences found in proteins that bind clathrin (19, 21, 33–35, 38, 39). The comparison delineates a conserved clathrin-box motif, a consensus noted in ref. 21. The clathrin box is surrounded in each of these proteins by unrelated residues.

12-residue peptide from the hinge of β3 (Fig. 1 *c* and *d*). We chose the β3-hinge, because the interacting sequence had been mapped most precisely (21); we demonstrate below that only the common features in the "clathrin box" of all these sequences determine the specificity of binding with clathrin.

The two structures we have determined are extremely similar, with the common motif in the clathrin box bound in the blade-1/blade-2 groove. We also have obtained apparently isomorphous crystals with the β1- and β2-hinge peptides of AP-1 and AP-2, consistent with the notion that they form a complex of similar structure (not shown). Sharing of the same site by arrestins and adaptors was unexpected, because previous studies had suggested that β-arrestin 2 and AP-2 would bind at different sites on the terminal domain (20). Clathrin-box motifs of highly conserved character are found in other proteins that interact with clathrin, such as AP-1, amphiphysin, epsin, AP180, and LAP (19, 21, 33–40) (Fig. 2). We conclude that all these proteins contact the same groove on clathrin.

The β-arrestin and β-hinge peptides have nearly identical bound conformations lining the groove between blades 1 and 2 of the β-propeller, with their amino-terminal ends near the "top" surface of the domain (Fig. 1 *b* and *d*). Only the central seven or eight residues in each peptide are ordered, and the key clathrin-box residues are LIEFE (373–377) in the β-arrestin 2 peptide and LLDLD (818–822) in the AP-3 β-hinge peptide (Figs. 1 and 2). The first and third residues in the clathrin-box motif augment blade 1 by engaging in two β-sheet-like hydrogen bonds with the exposed edge of strand d (Fig. 3). The amino-terminal part of the ordered peptide segment is stabilized further by hydrogen bonds between its backbone and the side chain of Q89. The leucine side chain of the initial residue in the clathrin box fits into a small hydrophobic pocket, and the side chains of the second and fourth residues, which are also hydrophobic, fit into a larger second pocket. The fifth, acidic residue of the clathrin box is flanked by R64 and K96. Thus, only the outwardly projecting, central residue of the motif lacks specific interactions, and the identity and charge of this residue are indeed not conserved, although it is always polar (Fig. 2).

The interactions just described account for all the conserved features of the clathrin box, and it is possible that many of the proteins in Fig. 2 have this motif as their only direct contact with clathrin. Arrestins, however, could have additional contacts beyond those visualized in the cocrystal, because preliminary experiments suggest that the β-arrestin 2 and the β1-, β2-, and β3-hinge peptides compete with the AP β2-chain hinge-ear fragment for binding to terminal domain, but not with intact

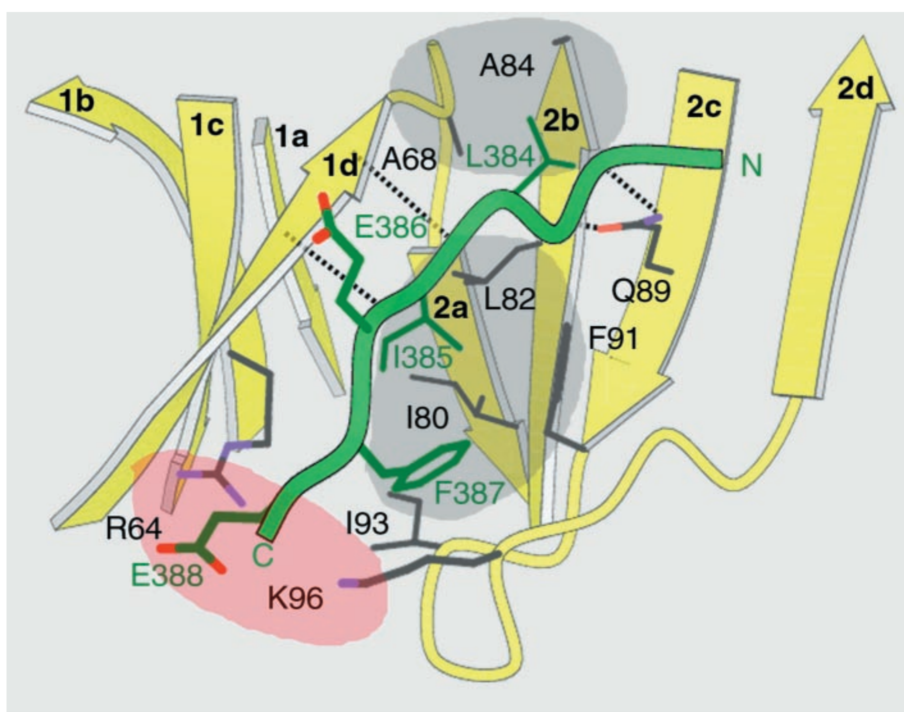

Fig. 3. Close-up view of the peptide-in-groove interactions between the clathrin box in the peptide of β-arrestin 2 and the clathrin-terminal domain. The residues that contribute to the interaction between the β-arrestin 2 peptide and the clathrin groove between blades 1 and 2 are labeled by single-letter code and by the positions in their respective sequences. The dashed lines show hydrogen bonds between the backbones of blade 1d and bound peptide and between peptide backbone and the side chain of clathrin Q89. The locations for the two hydrophobic (gray) and polar (rose) pockets in the clathrin groove are approximate.

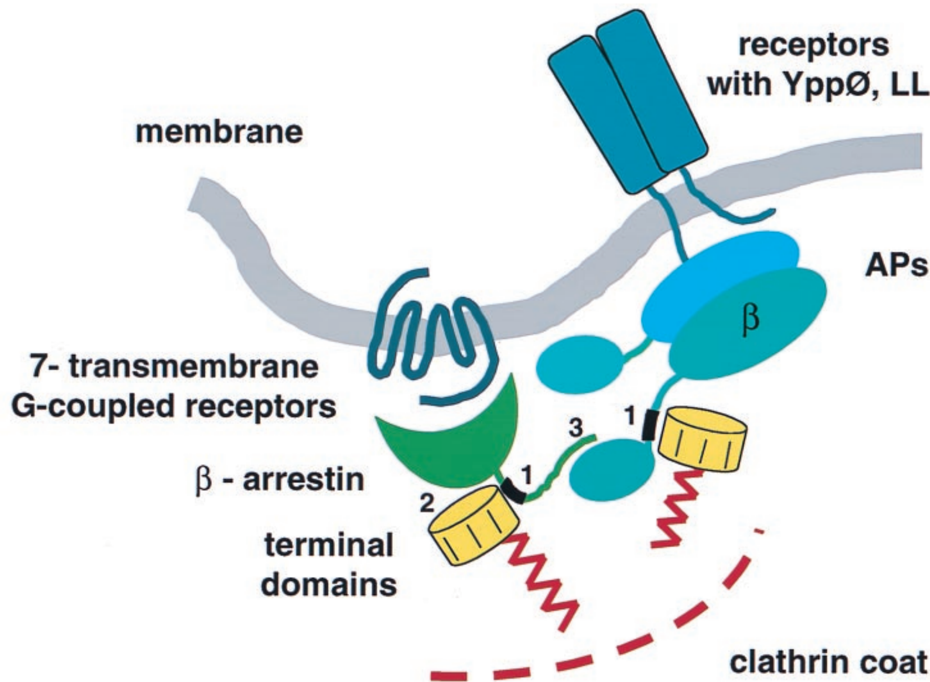

Fig. 4. Schematic representation of the network of contact arrays of target proteins and clathrin within a coat. Key features illustrated are: 1, the contact between the clathrin terminal domain (yellow) and the clathrin-box sequences (black) in β-arrestins (green) and the β-chain of AP complexes (blue); 2, a possible additional contact (see text) between β-arrestins and terminal domain; 3, a contact, inferred from mutational studies between the carboxyl-terminal part of β-arrestins and the β-subunit of AP-2. APs are generalized adaptors for sorting signals (YppØ, LL) in various receptors; β-arrestins are specific adaptors for seven-transmembrane, G protein-coupled receptors (dark blue). The membrane is shown in gray; the framework of the clathrin coat is shown in red.

CELL BIOLOGY

β-arrestin 1 (C. Brunner and T.K., unpublished data). Thus, the principal folded domain of β-arrestins may contact another part of the β-propeller in addition to the blade-1/blade-2 groove, for example, the surface that faces the membrane (see Fig. 4). We note that the β-propeller in the Gβ-subunit of heterotrimeric G-proteins accommodates a helix from the carboxyl terminus of the Gα-subunit along the groove between blades 1 and 7, but it also contacts Gα through loops on its top surface (1). That is, there are local interactions of a projecting peptide (in this case helical) with a single groove of the propeller ("peptide recognition") as well as extended interactions between surfaces of two compact domains ("surface recognition").

All the proteins known to interact with the clathrin-terminal domain contain a clathrin-box sequence (Fig. 2), and therefore we believe that they all bind similarly in the blade-1/blade-2 groove. In coated pits and vesicles, the "top" surface of the clathrin propeller faces the membrane (8), and the orientations of the β-arrestin 2 and AP β3-chain peptides in our structures correspond to the simplest path for a segment emanating from the C-terminal part of a membrane-anchored protein (Fig. 4). Can any of the other six grooves serve as sites for different motifs, not yet detected? The groove between blades 1 and 2 is the widest of the seven, largely because blade 2 is displaced outward from the axis of the propeller (evident in the view in Fig. 1 *b* and *d*) (2), and the β-arrestin and β-hinge peptides cover some of the contacts on strands c and d of blade 2 that otherwise would be covered by strand d of blade 1. A smaller relative displacement of blade 5 makes the groove between blades 4 and 5 the next widest, and it is the best candidate for a site to accommodate alternative peptide motifs. A recent structure of tachylectin-2, a five-blade β-propeller with multiple binding sites for *N*-acetylglucosamine, shows that each of its grooves is a carbohydrate-binding pocket for *N*-acetylglucosamine (41), with hydrogen bonds to the backbone groups of strand d.

β-Arrestins also interact with AP-2 complexes (42), and hence they may associate with clathrin by indirect links as well as by direct ones. Yeast two-hybrid studies suggest that the carboxyl-terminal segment of β-arrestin 2 interacts with the β2 chain of AP-2 (42). Likewise, amphiphysin, which contains a clathrin box (Fig. 2) by which it contacts clathrin, also appears to contact the ear of the α-chain of AP-2 (33, 43). Amphiphysin binds strongly to dynamin (44–46), and it is believed to link coat assembly with budding of the membrane. Thus, formation of a coated pit and loading of cargo are both likely to involve networks of contacts, each relatively weak, which cooperate to stabilize particular interactions at particular stages of the assembly cycle. A typical coated pit contains more than 100 clathrin-terminal domains, arrayed in a relatively dense layer near the membrane surface (8, 9) (Fig. 4). Even if a large number of proteins in the coated pit interact with the same groove of the clathrin propeller, they are unlikely to saturate all the available terminal domains. When these clathrin ligands also interact with each other, as suggested for β-arrestins or amphiphysin and AP complexes, they therefore still can cooperate, rather than compete, for inclusion in the same coated vesicle.

The clathrin-terminal domain is not the only example of a WD40 or WD40-like β-propeller that recognizes a defined peptide sequence. One particularly striking case is the protein known as h-βTrCP (47). This homologue of the yeast protein Cdc4 contains both an "F-box," which recruits it to the Skp1-Cullin-F-box (SCF) ubiquitination complex, and a WD40 domain, through which it recognizes substrates for the ubiquitin ligase. Specific targets for h-βTrCP include the NFκB inhibitor, IκB, β-catenin, and the HIV protein Vpu (which, in turn, recruits CD4) (47–52). Phosphorylation of two serines, separated by three residues, in IκBα, β-catenin, and Vpu allows

binding to the WD40 domain, resulting in polyubiquitination and, ultimately, in proteosomal degradation. The common six-residue sequence in the IkB proteins, β-catenin, and Vpu is DpSGXXpS (pS denotes phosphoserine; X, any amino acid). We suggest that the WD40 domain of h-βTrCP has a groove between two of its blades that accepts the diphosphorylated segment of the substrate. Peptide-in-groove recognition, as seen in the clathrin interactions described here, is likely to be a general property of WD40 proteins and β-propellers.

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