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Commentary

Wrapping the package

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The membranes of living cells are in a state of dizzying flux. Lipid vesicles, often containing receptors and wrapped generally in protein coats, shuttle between cellular compartments at such a rate that, for example, the substance of the plasma membrane is typically turned over in less than an hour. Of the known classes of vesicles, the most extensively studied, by reason of their abundance and ease of preparation, are the clathrin-coated vesicles. These are the vehicles through which cell-surface receptors are internalized and delivered to the endosomal compartment, and they also transport lysosomal enzymes from the trans-Golgi network to the endosomes (1).

A group of proteins dubbed adaptors secure the vesicle's cargo (e.g., receptors) to the clathrin coat. Different adaptors are specific to particular transport routes. The adaptor of endocytic clathrin-coated vesicles is called AP-2 (1). It is made up of four polypeptide chains designated α, $\beta 2$, $\mu 2$, and $\sigma 2$. Electron micrographs of AP-2 reveal a brick-like core with two globular appendages that are connected to the core by flexible stalks (2) [see also Fig. 1 in Traub *et al.* (3)]. The α and $\beta 2$ subunits are polypeptides of $\approx$100 kDa, each consisting of three structural domains: a 60- to 70-kDa N-terminal domain, followed by a short flexible linker and a $\approx$30-kDa C-terminal domain (4). The N-terminal domains, together with $\mu 2$ and $\sigma 2$ subunits, form the core, whereas the 30-kDa C-terminal domains correspond to the respective appendages. AP-2 interacts with clathrin through the $\beta 2$ linker segment and possibly also through the N-terminal α-adaptin domain (5, 6). The $\mu 2$ subunit binds to tyrosine-based internalization motifs, which are found in many cytosolic receptor tails (7). The α-appendage domain, on the other hand, seems to be the common locus for the attachment of an ever-growing number of endocytic accessory proteins, most of which were shown to be essential for clathrin coat formation (8–12). These include eps15, epsin, amphyphysin I and II, dynamin, AP180, and the most recent addition, auxilin (13). They all have in common one or more copies of the motif DPF/W, which occurs in the respective α-appendage-binding domains (14, 15). Most of the binding partners of the α-appendage were purified from cytosol in so-called pull-down experiments, in which an immobilized expressed glutathione *S*-transferase-tagged form of the appendage was used as the affinity matrix (8). Transfection experiments with dominant-negative constructs suggest that eps15 interferes with the recruitment of AP-2 to the plasma membrane and thus blocks receptor-mediated endocytosis (11). Because the primary structure of the α-appendage afforded no clues to the manner of its interaction with the endocytic accessory proteins, two laboratories, one in Cambridge, England, the other in the Midwest, were moved to attempt its crystallization. Both succeeded, and the resulting structures appear in this issue of the *Proceedings* (3) and in a recent issue of *Cell* (13).

The two structures are reassuringly alike: the α-appendage folds into two tightly packed subdomains with no segmental mobility. The N-terminal subdomain (S701–F825) forms a two-sheet β-sandwich, comprising eight antiparallel β-strands. It has its structural precedent in the immunoglobulin superfamily fold. The C-terminal subdomain (F826–F938) consists of a slightly curved β-sheet, supported by two underlying α-helices, whereas a third helix crosses the platform-like sheet. Traub *et al.* (3) remark on the similarity of this structure to that of the yeast TATA-box-binding protein.

To identify likely sites for protein–protein interactions on the α-appendage domain, Traub *et al.* (3) scanned the surface for areas containing clusters of evolutionarily conserved residues. With the same goal in mind, the Cambridge group (13) used an algorithm to detect surface patches of hydrophobic side chains. Both approaches homed onto a solvent-exposed hydrophobic pocket on the C-terminal platform subdomain, lined with a set of invariant polar residues. As is commonly the case, the combination of hydrophobic and polar residues provides the affinity and specificity of the ligand interactions.

To demonstrate directly the role of the platform domain in ligand binding, both groups resorted to systematic mutation of hydrophobic residues that form the pocket and of nearby polar residues. The effect of the mutations on ligand binding was explored by way of pull-down binding experiments. Given the qualitative nature of the binding assay, the data obtained by the two groups are remarkably congruent and agree in concluding that the site for attachment of the endocytic accessory proteins eps15, epsin, amphyphysin I/II, dynamin, AP180, and auxilin is indeed in the platform domain. W840 turns out to be the most critical single residue, and its substitution by alanine annihilates all recognized binding activities (13). On the basis of the differential effects of several mutations and double mutations on ligand binding, the DPF/W motif-containing proteins fall into two groups, the first comprising amphiphysin and AP180, the second eps15, epsin, and auxilin. Unlike the wild-type appendage, mutants with impaired binding to accessory proteins do not inhibit efficiently transferrin uptake when transfected into COS cells (13). Overexpression of mutant proteins with reduced or no *in vitro* binding to certain endocytic accessory proteins is more informative than the overexpression of wild-type protein, because it addresses directly the function of the binding partners whose binding site on the appendage had been destroyed by mutagenesis.

The importance of the DPF/W sequence motif in the interaction between accessory proteins and the appendage was directly shown by Owen and coworkers with synthetic peptides containing either the DPF or the DPW motif (13). In pull-down experiments, both peptides interfered with the association of all known binding partners to the α-appendage. The dissociation constant of the interaction between peptide and appendage suggest a relatively low affinity, but it is not much lower than that of glutathione *S*-transferase-fusion proteins containing either the DPW domain of epsin or the DPF domain of eps15 (12). In general, the interactions between the components of the clathrin transport machinery seem to be characterized by low pairwise affinities. Examples are the interaction between the tyrosine-based internalization motifs with the $\mu 2$ adaptor subunit (1–10 μM) (16), that of clathrin

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with the linker/hinge segment of the β-adaptin, and those between EH domains and NPF motifs (≈500 μM) (17). This low affinity is probably also the reason why one cannot trap complexes between individual clathrin triskelia and either of the adaptor complexes, auxilin or AP 180. These weak interactions of the ligands with clathrin nevertheless suffice to induce assembly of clathrin into cages, which then bind with high affinity to the aforementioned coat proteins. Thus it seems that multiple contacts, which are permitted only in the assembled state, are required if a stable structure is to form. The genesis of a clathrin-coated transport vesicle is a dynamic event requiring not only rearrangements of the lattice, but also transitory interactions with the endocytic accessory proteins. Epsin, eps15, amphiphysin, and dynamin are clearly required for coat formation, but none is a component of clathrin-coated vesicles. The advantage of a cooperative system of weak interactions is the ease with which a small environmental perturbation can tilt protein complexes in the direction of assembly or disassembly.

All the known coat and accessory proteins form a large cytosolic pool, implying that when dispersed in the cytosol, they do not extensively interact with each other. There is evidence that many of them are phosphorylated in these circumstances, and that on dephosphorylation they can be readily recruited to the immobilized α-appendage or, for that matter, to other suitable domains of endocytic accessory proteins (18). In any case, it seems that immobilization of an interaction domain on a surface—an agarose bead or the plasma membrane—is crucial for recruiting other members of the endocytic machinery. Thus the migration of a key cytosolic component to a membrane could lead to the rapid self-assembly of a coat and capturing of the cargo. It is unlikely that this component is AP-2, because eps15 recruitment to the plasma membrane is evidently in fact a prerequisite to AP-2 binding (11). It has been suggested that cytosolic eps15 is already constitutively associated with AP-2, but if so the association can only be weak, because free eps15 is readily captured from cytosol in pull-down experiments with the immobilized α-appendage. Thus it seems more likely that only membrane-bound eps15 binds cytosolic AP-2 efficiently. But which component recruits eps15 to the plasma membrane? A possible candidate is the protein intersectin, which was recently reported to bind eps15. Some of the many splice variants of intersectin contain PH and C2 domains, which could mediate its association with membranes (19).

It is at first sight surprising that the protein auxilin is one of the proteins that associate with the appendage-domain of AP-2 (13). Auxilin acts as a cofactor with Hsc70 in the release of clathrin coats from the vesicle membrane (20). It will be interesting to determine when and with what consequences it associates with AP-2 in cells. Its binding to the α-appendage could betoken a function for auxilin in the release of adaptors from the vesicle membrane, but the only experimental evidence for this scenario is scant (21). Another possibility is that auxilin attaches to the clathrin coat in early stages of assembly and then cooperates with Hsc70 in the specific disruption of protein–protein interactions that may be required for coat growth and its reorganization during budding.

Defining the sequence of events that culminates in the formation of the clathrin-coat assembly will be a formidable task. The next step could well rely on electron microscopy to establish which steps in coated vesicle assembly are made to fail by the dominant-negative accessory proteins.

1. Schmid, S. L. (1997) *Annu. Rev. Biochem.* **66,** 511–548.
2. Heuser, J. E. & Keen, J. (1988) *J. Cell Biol.* **107,** 877–886.
3. Traub, L. M., Downs, M. A., Westrich, J. L. & Fremont, D. H. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8907–8912.
4. Kirchhausen, T., Nathanson, K. L., Matsui, W., Vaisberg, A., Chow, E. P., Burne, C., Keen, J. H. & Davis, A. E. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2612–2616.
5. Shih, W., Gallusser, A. & Kirchhausen, T. (1995) *J. Biol. Chem.* **270,** 31083–31090.
6. Goodman, O. B., Jr. & Keen, J. H. (1995) *J. Biol. Chem.* **270,** 23768–23773.
7. Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T. & Bonifacino, J. S. (1995) *Science* **269,** 1872–1875.
8. Wang, L. H., Sudhof, T. C. & Anderson, R. G. (1995) *J. Biol. Chem.* **270,** 10079–10083.
9. Benmerah, A., Gagnon, J., Begue, B., Megarbane, B., Dautry-Varsat, A. & Cerf-Bensussan, N. (1995) *J. Cell Biol.* **131,** 1831–1838.
10. Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A. & Cerf-Bensussan, N. (1998) *J. Cell Biol.* **140,** 1055–1062.
11. Benmerah, A., Bayrou, M., Cerf-Bensussan, N. & Dautry-Varsat, A. (1999*) J. Cell Sci*. **112,** 1303–1311.
12. Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P. & De Camilli, P. (1998) *Nature (London)* **394,** 793–797.
13. Owen, D. J., Vallis, Y., Noble, M. E., Hunter, J. B., Dafforn, T. R., Evans, P. R. & McMahon, H. T. (1999) *Cell* **97,** 805–815.
14. Benmerah, A., Begue, B., Dautry-Varsat, A. & Cerf-Bensussan, N. (1996) *J. Biol. Chem.* **271,** 12111–12116.
15. Iannolo, G., Salcini, A. E., Gaidarov, I., Goodman, O. B., Jr., Baulida, J., Carpenter, G., Pelicci, P. G., Di Fiore, P. P. & Keen, J. H. (1997*) Cancer Res.* **57,** 240–245.
16. Rapoport, I., Miyazaki, M., Boll, W., Duckworth, B., Cantley, L. C., Shoelson, S. & Kirchhausen, T. (1997) *EMBO J.* **16,** 2240–2250.
17. de Beer, T., Carter, R. E., Lobel-Rice, K. E., Sorkin, A. & Overduin, M. (1998) *Science* **281,** 1357–1360.
18. Slepnev, V. I., Ochoa, G. C., Butler, M. H., Grabs, D. & Camilli, P. D. (1998) *Science* **281,** 821–824.
19. Sengar, A. S., Wang, W., Bishay, J., Cohen, S. & Egan, S. E. (1999) *EMBO J.* **18,** 1159–1171.
20. Ungewickell, E., Ungewickell, H., Holstein, S. E., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L. E. & Eisenberg, E. (1995) *Nature (London)* **378,** 632–635.
21. Hannan, L. A., Newmyer, S. L. & Schmid, S. L. (1998) *Mol. Biol. Cell* **9,** 2217–2229.