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ADAPTORS FOR CLATHRIN-MEDIATED TRAFFIC

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■ **Abstract** Clathrin-based systems are responsible for a large portion of vesicular traffic originating from the plasma membrane and the *trans*-Golgi network that reaches the endosomal compartment. The assembly of cytosolic clathrin forms the scaffold required for the local deformation of the membrane and for the formation of coated pits and vesicles. In this process, clathrin interacts in a coordinated fashion with a large number of protein partners. A subset designated clathrin adaptors links integral membrane proteins to the clathrin coat, a process that results in the recruitment of specific cargo proteins to the budding vesicle. This review focuses on the most recent advances dealing with the molecular basis for sorting by clathrin adaptors.

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INTRODUCTION

Transport between membrane-bound compartments in the endocytic and secretory pathways is mediated by vesicles that carry cargo protein. At the donor membrane, specialized invaginations or pits form, which upon budding create the carrier vesicle. A number of cytosolic proteins are involved in a complex and highly coordinated process. There is selective capture of the cargo molecules at the budding site, together with the recruitment of regulatory and structural proteins required for local deformation and fission of the membrane pit and for delivery and fusion of the resulting vesicle to the target membrane. Clathrin-based systems are responsible for a large fraction of the vesicular traffic that reaches the endosomal compartment, originating from the plasma membrane or from the *trans*-Golgi network (TGN). During vesiculation, cytosolic clathrin triskelions assemble into a coat that drives membrane deformation. Clathrin also contacts a number of other proteins, some of which are directly involved in the recruitment of cargo. They are referred to as adaptors because they link clathrin coats and cargo sorting. This designation represents an oversimplification, however, because the β-arrestin-like adaptors (Lohse et al 1990, Luttrell et al 1999) and the clathrin adaptor-protein (AP) complexes clearly have other important roles. There are also proteins such as AP180/CALM (Morris et al 1993, Murphy et al 1991, Ye & Lafer 1995b), epsin 2, and amphyphysin (Ramjaun & McPherson 1998) that appear to interact with clathrin or that contain a sequence with the potential to interact with clathrin, but whose function is less well understood. Until their function is clearly established in the clathrin pathway, we shall describe them more generally as clathrin partners.

Since the last time adaptor complexes were reviewed (Hirst & Robinson 1998, Kirchhausen et al 1997, Lewin & Mellman 1998, Schmid 1997), enormous progress has occurred. The molecular basis for sorting signal recognition by clathrin adaptors has been determined; novel adaptors have been identified, such as members of the β-arrestin family (Goodman et al 1996) and the AP-3 (Dell'Angelica et al 1997, Panek et al 1997, Simpson et al 1997) and AP-4 adaptor complexes (Dell'Angelica et al 1999a), which resemble AP-1 and AP-2; and atomic structures of components of the clathrin pathway have been determined, including portions of clathrin (Ter Haar et al 1998), arrestins (E Ter Haar, personal communication), and APs (Owen & Evans 1998, Traub et al 1999). This review focuses on the most recent of these advances.

MONOMERIC ADAPTORS

β-Arrestins

The β2-adrenergic receptor, a model member of the seven-helix membrane-spanning, G-coupled receptor family is rapidly activated upon binding its ligand, thereby eliciting a range of intracellular signaling events. One of the downstream consequences is a regulatory mechanism that causes activated receptors to be

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removed efficiently from the cell surface by clathrin-coated vesicles directed to the endosomal compartment (Ferguson et al 1996, Freedman et al 1995, Goodman et al 1996, Lohse et al 1990, Tolbert & Lameh 1996, von Zastrow & Kobilka 1992). This relocation of the activated β2-adrenergic receptor appears to have three purposes: It allows the activated receptor to interact with other proteins in the endosomal membrane (Luttrell et al 1999), it might allow for the recycling of desensitized receptors back to the plasma membrane as competent receptors (von Zastrow & Kobilka 1992), and it allows for eventual shutdown of the entire β2-adrenergic receptor signaling pathway by directing the activated receptors to lysosomes for destruction.

Activated β2-adrenergic receptors recruit the proteins β-arrestin1 (arrestin2) and β-arrestin2 (arrestin3) to their cytoplasmic face. These relatively simple adaptors link the receptor-arrestin complex to the clathrin assembly that underlies an invaginated clathrin-coated pit. β-arrestin1 and β-arrestin2 are monomeric proteins of $\sim$45 kDa. The visual α-arrestin, closely related to β-arrestins in sequence, downregulates rhodopsin signaling by a different mechanism (disengaging the trimeric G protein), and it does not act as a clathrin adaptor (Dolph et al 1993, Goodman et al 1996). X-ray crystallographic analysis of α-arrestin reveals that it has a globular N-terminal core of about 368 residues and a partially disordered 36 residue C-terminal tail (Granzin et al 1998, Hirsch et al 1999). The sequence of the globular core is highly conserved among the three arrestins, and the three proteins are therefore likely to have a similar fold. The tail is somewhat less conserved in sequence than the globular core, but we expect that this region is also likely to be disordered in the β-arrestins. In β-arrestin1 and β-arrestin2 this region not only contacts the cytosolic portion of the β-adrenergic receptor but also binds src (Luttrell et al 1999), the phosphate groups of inositol rings in inositol hexaphosphate (IP_6), and the lipid-bound inositol phosphates PtdIns3,4-P_2 and PtdIns4,5-P_2 (Gaidarov et al 1999), presumed to facilitate the recruitment of arrestins to the membrane interface.

The C-terminal tails of β-arrestin1 and β-arrestin2 are required for downregulation of activated β2-receptors, and two regions have been mapped that associate with clathrin coat components. A short sequence, in the flexible C-terminal tail, LIEF/LE (starting at residue 374 of β-arrestin2), is required for clathrin binding (Krupnick et al 1997). This element, referred to as the clathrin box, is not present in α-arrestin, consistent with its lack of clathrin adaptor function. A region between residues 378 and 410 appears to mediate association of β-arrestins with the β2-chain of AP-2 complexes in the yeast-two-hybrid system (Laporte et al 1999). Another region around residues 350–371, at the interface between the globular core and the disordered tail, also contributes to the ability of arrestin to downregulate β2-receptors (Orsini & Benovic 1998), suggesting that this region may also be involved in clathrin binding. Overexpression of β-arrestin2 lacking both regions does not support internalization of activated β2-adrenergic receptors. Unexpectedly, it has been observed that overexpression of β-arrestin2 lacking the clathrin binding site but containing the AP-2 binding site still promotes receptor endocytosis

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(Laporte et al 1999). It is possible that at normal levels of β-arrestin expression both interactions are needed, e.g. for pre-clustering of activated β2-adrenergic receptors/β-arrestin2 complexes with AP-2 to facilitate transfer to an assembling coated pit.

The contact site for the β-arrestins is found in the N-terminal domain of clathrin (Goodman et al 1997). Each clathrin molecule has three bent legs, each with a globular, N-terminal domain at its tip (Kirchhausen 1993). In a coated pit or coated vesicle, the N-terminal domains project toward the underlying membrane in the clathrin coat, and therefore are plausible sites for interaction with other proteins in, or just below, the membrane (Heuser & Kirchhausen 1985, Smith et al 1998, Vigers et al 1986, Musacchio et al 1999). The recently determined X-ray crystal structure of the clathrin terminal domain has allowed for mapping the contact area between clathrin and the arrestins in more detail (Ter Haar et al 1998). The clathrin terminal domain has the shape of a seven-blade β-propeller. Mutagenesis studies show that residues now known to lie in the groove between blades one and two of the propeller are required to interact with the clathrin-binding box of the β-arrestins (Goodman et al 1997). We recently obtained the X-ray crystal structure of a complex containing the terminal domain of clathrin and a 73-mer peptide, including the clathrin box LIEFE of β-arrestin2 (E Ter Haar, SC Harrison & T Kirchhausen, submitted for publication). The clathrin box peptide adopts a stable conformation, probably imposed by binding to the folded terminal domain. Related clathrin box sequences have been detected in other proteins such as the clathrin adaptor complexes (APs) (Dell'Angelica et al 1998, Shih et al 1995), amphyphysin (Ramjaun & McPherson 1998), and epsin 2. In each case, the clathrin box sequence is required for the interaction with clathrin.

Although β-arrestins can bind clathrin, they do not drive clathrin coat assembly in in vitro reactions designed to screen for coat-formation activity (Goodman et al 1996). Moreover, overexpression in transfected cells of a dominant-negative form of arrestin, which binds clathrin but not β2-receptors, prevents downregulation of β2-receptors, as expected, but does not prevent endocytosis of transferrin (used as a generic probe to measure perturbations in the clathrin-dependent uptake pathway). Activated β2-adrenergic receptors form complexes with β-arrestins that can recruit AP-2 adaptors to the plasma membrane (Laporte et al 1999). Thus arrestins appear to act as adaptors linking cargo recruitment to a coated pit with the potential to indirectly influence the nucleation or regulation of coated pit formation.

MULTIMERIC ADAPTORS

The Adaptor Complexes or APs

A second group of adaptors constitute a family of heterotetrameric complexes, ~300 kDa in size, which are found to varying extents in all nucleated cells from yeast to humans. The founding members are the mammalian AP-1 complex, specific for traffic from the *trans*-Golgi network to the endosome, and two AP-2

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adaptor complexes specific for traffic originating at the plasma membrane but also to the endosome (Ahle et al 1988, Robinson & Pearse 1986). A second mammalian AP-1 complex, specific to the endosomal compartment, has just been identified (Lewin & Mellman 1998, Takatsu et al 1998). The fifth family member, AP-3, is mostly associated with endosomal membranes (Dell'Angelica et al 1997, Simpson et al 1997; reviewed in Hirst & Robinson 1998, Odorizzi et al 1998); thus far two slightly related forms of these complexes have been identified. It has not yet been definitively established if they form clathrin coats in vivo. The seventh family member, AP-4, is associated with TGN-membranes (Dell'Angelica et al 1999a).

The AP-1 and AP-2 complexes interact directly with clathrin through a clathrin box sequence in its β chain (Gallusser & Kirchhausen 1993, Shih et al 1995) and induce the assembly of clathrin-AP coats in vitro (Ahle & Ungewickell 1989, Pearse & Robinson 1984, Zaremba & Keen 1983). Mammalian AP-3 contains a clathrin box sequence (Dell'Angelica et al 1998), and it can also induce coat formation in vitro, albeit inefficiently (I Rapoport & T Kirchhausen, unpublished observation); yeast AP-3 does not contain a clathrin box sequence and does not appear to interact with clathrin (Cowles et al 1997, Stepp et al 1997, Vowels & Payne 1998; G Payne, personal communication). The coat assembly function has been assumed to reflect a need for APs in recruiting clathrin to the membrane, either to the nucleation site as the decision to form a pit is made, or to the growing edges of the lattice, as the coat assembles (Hirst & Robinson 1998, Kirchhausen 1993, Kirchhausen et al 1997, Robinson 1994, Schmid 1997, Shih et al 1995). However, some recent genetic experiments in yeast, described below, are not consistent with this hypothesis (Huang et al 1999; G Payne, personal communication). A second and by now well-established function of APs is to recognize the sorting signals present in the cytosolic tails of those membrane-bound proteins that are recruited to coated pits and vesicles (Pearse & Robinson 1990). The interaction between APs and sorting signals, like the interaction between clathrin and the APs, is based on the recognition of a short peptide by a folded protein domain (Boll et al 1996, Ohno et al 1995, Owen & Evans 1998, Rapoport et al 1998; E Ter Haar, SC Harrison & T Kirchhausen, submitted for publication). Thus the cargo molecule/AP/clathrin complex is strung together by flexible peptide extensions from the cargo molecule to the AP, and from the AP to clathrin.

Composition

The AP complexes are made of four different types of adaptin chains (Hirst & Robinson 1998, Keen 1990, Kirchhausen 1993, Pearse & Robinson 1990, Schmid 1997) (Figure 1, see color insert). Each complex contains two large chains: $\gamma 1$ and $\beta 1$ for AP-1, α and $\beta 2$ for AP-2, δ and $\beta 3$ for AP-3, and ε and $\beta 4$ for AP-4 (Figure 2, see color insert). In addition, each complex contains one medium chain, $\mu 1$, $\mu 2$, $\mu 3$, or $\mu 4$, for AP-1, AP-2, AP-3, or AP-4, respectively, and one small chain, $\sigma 1$, $\sigma 2$, $\sigma 3$, or $\sigma 4$. The large chains are $\sim$100 kDa in size, whereas the medium and small chains are $\sim$50 kDa and $\sim$20 kDa, respectively. Comparison

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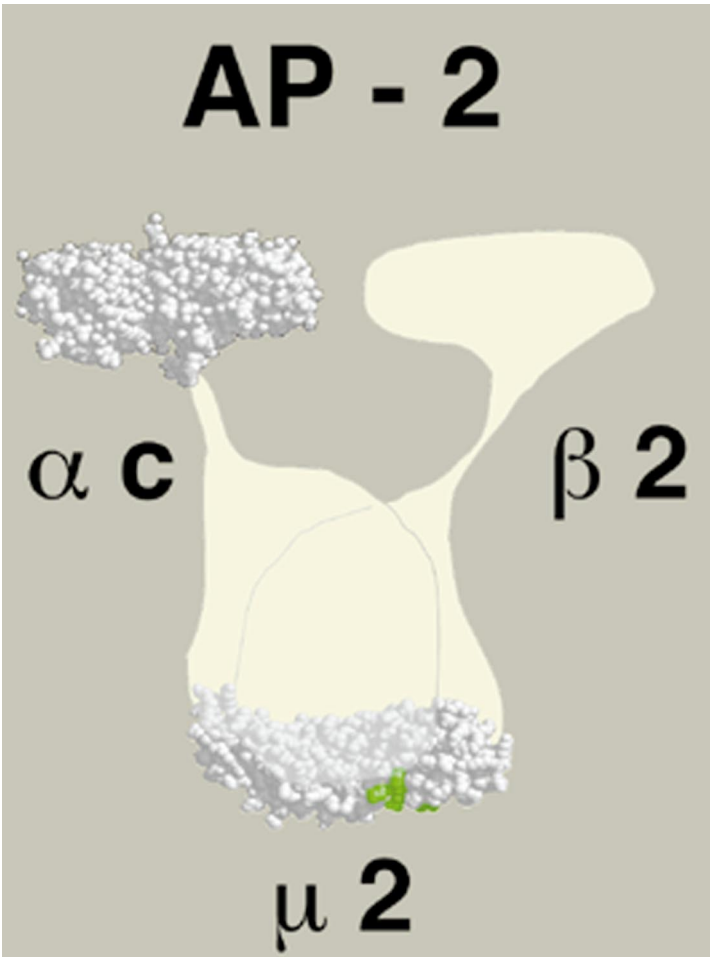

Figure 1 Schematic representation of the plasma membrane multimeric adaptor AP-2. The surface representations correspond to the structures of the α-ear and of a fragment of μ2, bound to a peptide bearing the YQRL sorting signal of TGN38 (green), which have been determined by X-ray crystallography. The precise location of the AP subunits is not known.

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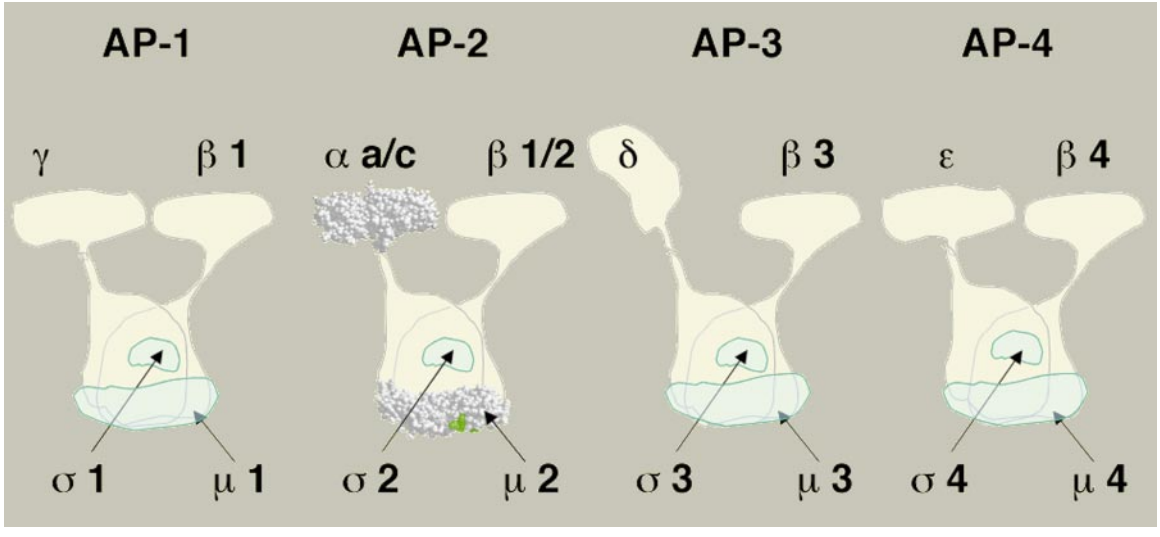

Figure 2 Schematic representation of the multimeric adaptors AP-1, AP-2, AP-3, and AP-4. The surface representations correspond to the structures of the α-ear and the fragment of $\mu 2$ bound to the sorting signal of TGN38 (green), determined by X-ray crystallography.

of the protein sequences of β, μ, or σ chains among AP adaptors shows that their primary structures are highly conserved (50–80% identity). In contrast, the α, δ, γ, and ε chains, although clearly related, diverge significantly from each other, with only ~25% overall identity. The α and β chains from mammalian cells also display neuronal-specific splicing, but the role of this modification is not known.

Shape and Domain Organization

As AP-2 is the most abundant adaptor, and because the similarities among AP-1, AP-2, AP-3, and AP-4 are extensive, most studies to determine the domain organization of the adaptors have been done with AP-2. Electron microscopic visualization of rotary-shadowed AP-2 adaptors shows images consisting of an $90 \times 70 \times 70$ Å globular core or head flanked by two approximately 30 Å globular appendages or ears (Heuser & Keen 1988). The stalk or hinge that joins the head to the ears is thought to be flexible because the disposition of the ears with respect to the head can vary significantly in different images of AP-2. The head and ears of AP-1 and AP-2 are relatively resistant to enzymatic proteolysis, whereas the hinges are very sensitive, which has led, upon protease treatment, to the separation of head and ears (Schroder & Ungewickell 1991, Zaremba & Keen 1985). The ears correspond to the C terminus of the large chains, whereas the head contains the remaining N-terminal two thirds of the large chains plus the medium and small chains (Kirchhausen et al 1989) (see Figure 1 for cleavage pattern). The medium and small chains are well protected from proteolysis in the complex, indicating that they do not have flexible protrusions. At least one area of the μ chains must be readily accessible to other proteins, however, because this subunit contains the binding site for the tyrosine-based cargo-sorting motif Yppϕ (Ohno et al 1995, Owen & Evans 1998).

Chemical cross-linking of AP-2 showed that the α chain is in close contact with the β chain and that the large chains are in contact with the μ and σ chains, but the cross-linking did not indicate any points of contact between the μ and σ chains (Pearse & Robinson 1984, Virshup & Bennett 1988). Yeast two-hybrid experiments have recently been used to study these inter-subunit contacts in more detail, showing that α and γ chains interact with β chains and that β chains also binds μ chains (Page & Robinson 1995). Finally, immunoprecipitation studies of native APs in cells expressing chimeric proteins that contained different proportions of α and γ chains showed that ~130 amino acids at the N terminus of α/γ determine the specific interaction with the appropriate μ and σ chains (Page & Robinson 1995). This experiment does not, however, answer the question of whether μ and σ chains make direct contact with each other. A definitive answer will require the high-resolution determination of the structure of an AP head.

Atomic Structure of the Ear Domain of the α Chain

The structure of the C-terminal portion (residues 692 to 938) of the αC chain of AP-2 has been determined by X-ray crystallography (Traub et al 1999, Owen

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et al 1999) (Figure 3, see color insert). It contains two domains: an eight-strand β-barrel linked to an α/β domain based on a four-strand antiparallel sheet. There is a noticeable cleft between the two domains, which might represent a binding site for a peptide from a partner protein. The sequences of α- and γ-chains are closely related, especially in their N-terminal regions, and it is likely that the two proteins have a very similar fold (Robinson 1989, 1990).

Recognition of Sorting Signals

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Genetic and cell biological experiments have clearly established that intracellular traffic of many membrane-bound proteins requires sequences facing the cytoplasm. In many instances the sorting information is encoded in short peptide motifs, typically 4–6 amino acids, which are referred to as sorting signals. These motifs determine which vesicular traffic pathway is used to transport a particular molecule and hence determine its final destination. In other cases, sorting requires a larger portion of the cytosolic side of the cargo protein to be transported, presumably indicating that a folded domain in the cargo contains the sorting information. In some cases, mono-ubiquitination of the cytosolic side of a membrane protein also seems to induce internalization from the plasma membrane, although the mechanism is unclear.

Sorting signals are clearly important for sorting in the clathrin-dependent (Kirchhausen et al 1997, Marks et al 1997) and COPI- and COPII-dependent pathways (Cosson & Letourneur 1997, Lowe & Kreis 1998) and may well be important in other vesicular trafficking. For example, Tip47 is a 47-kDa soluble protein that recognizes a phenylalanine/tryptophan-based sorting signal in the cytoplasmic tail of the mannose 6 phosphate receptor and is involved in its traffic from the endosome to the TGN (Diaz & Pfeffer 1998); however, no sorting signals have been identified for the vesicles that return proteins from the endosome to the plasma membrane. Here we focus on the sorting signals used by the clathrin pathway, which are now beginning to be understood at a molecular level.

The NPXY Motif The endocytic sorting signal in the tail of the LDL receptor was the first sorting motif identified for the clathrin pathway (Anderson et al 1977, Lehrman et al 1985). This motif has the form NPXY (where N, P, and Y are asparagine, proline and tyrosine, and X can be any amino acid). The NPXY motif is both necessary and sufficient for internalization, and the extent of endocytosis for a protein that contains the NPXY motif in its cytoplasmic tail does not depend on the sequence context in which the motif is found. Although this motif was the first to be identified, it is perhaps the least well understood of the clathrin pathway sorting signals. The interaction of the sorting machinery with this motif, presented either in a short peptide or in the complete cytoplasmic tail of the LDL receptor, is extremely weak. Interactions with K_ds in the low millimolar range have been observed with both the terminal domain of clathrin (Kibbey et al 1998) and with APs (Pearse 1988; I Rapoport, personal communication), but it is unclear whether

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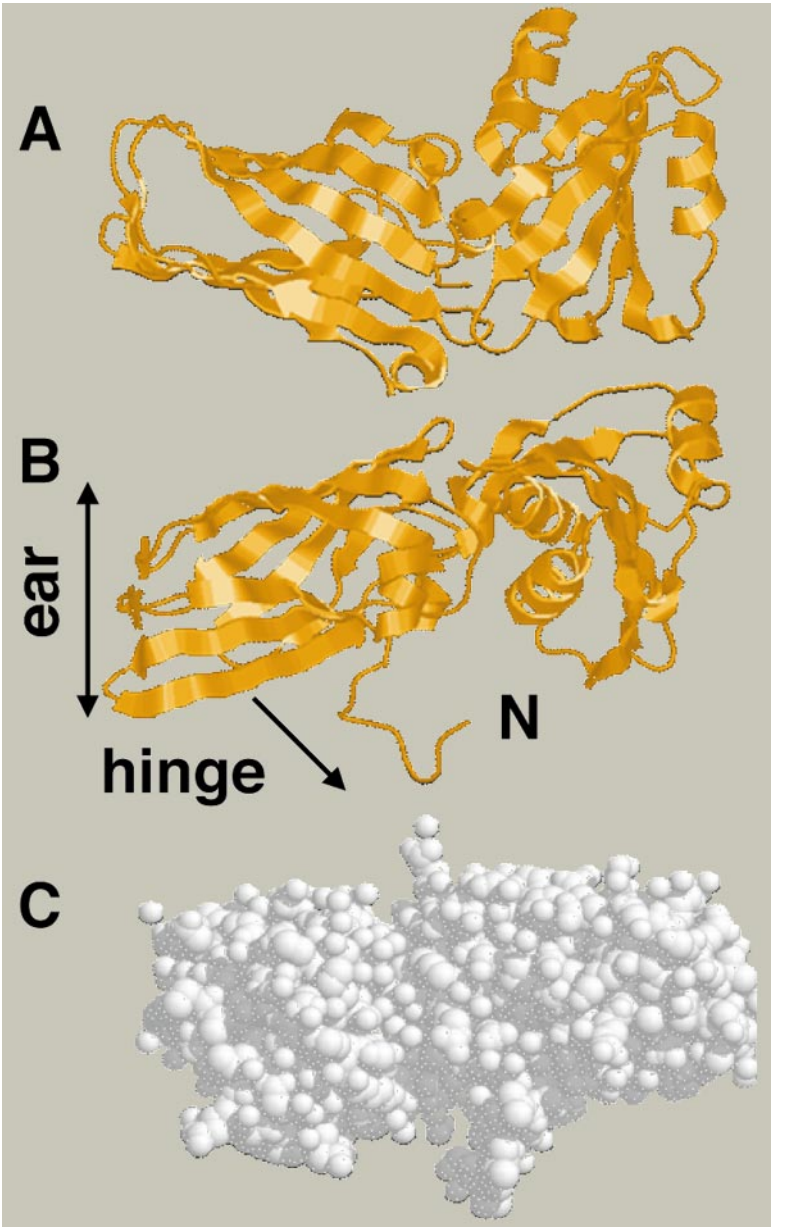

Figure 3 Atomic structure of the C-terminal ear of the α-C chain of AP-2. The fragment is view from the top in (*A*), and from the side in (*B*) and (*C*). The segments corresponding to the ear and part of the hinge are indicated.

these interactions are specific. Interestingly, the NPXY motif is extremely rarely used as a sorting signal, and it may be that in fact it does not recognize a general coat component such as the APs or clathrin, but instead requires a specialized adaptor, perhaps akin to the β-arrestins.

The Yppϕ Motif A second type of sorting signal used in clathrin-dependent traffic was first identified in the cytosolic tail of the transferrin receptor, where it is used to direct its uptake from the plasma membrane (Collawn et al 1990, Trowbridge et al 1993). It has the general form Yppϕ, where Y is tyrosine, p is a polar residue, and ϕ is a residue with a bulky hydrophobic side chain. The Yppϕ motif is found in a large number of membrane proteins and is used more broadly than the NPXY motif, not only as an endocytic motif but also to direct traffic within the endosomal and the secretory pathways (Kirchhausen et al 1997, Marks et al 1997). From recent experiments carried out in several laboratories, using a wide range of approaches, it is now well established that the Yppϕ motif is a peptide-type sorting signal specifically recognized by the μ-chain of APs.

The μ-Chains Recognize the Yppϕ Motif The first strong indication that AP-2 complexes recognize Yppϕ signals came from the realization that EGF receptors (EGFR) obtained from cells stimulated with EGF co-immunoprecipitate with AP-2 adaptors (Sorkin & Carpenter 1993). Upon binding to EGF, EGFR rapidly concentrates in clathrin-coated pits and is internalized from the plasma membrane (Carpentier et al 1982, Hanover et al 1984). It was therefore satisfying to find that EGFR associates with AP-2 only after the addition of EGF. Soon thereafter it was shown by in vivo and in vitro experiments that the EGFR/AP-2 association is direct and stoichiometric and requires the endocytic YRAL motif of EGFR (Boll et al 1995; Nesterov et al 1995; Sorkin et al 1995, 1996).

Further evidence came from a yeast-two hybrid experiment that identified an interaction between the μ2 chain of AP-2 and Yppϕ-containing peptides (Ohno et al 1995). These experiments used the Yppϕ motif from the membrane protein TGN38, which normally resides in the TGN but during biosynthesis traffics through the TGN, continues to the plasma membrane and returns back to the TGN via the clathrin-dependent pathway (Humphrey et al 1993). Its sorting signal, YQRL, is found at the C-terminal end of its 34-amino acid cytosolic tail, following the sequence SD. Initially, a short peptide made of a triplet repeat of SDYQRL was used as a bait in a yeast two-hybrid screen against a complementary DNA (cDNA) prey library from mouse spleen cells. Two interacting clones were identified from approximately 2.5×10^6 clones, corresponding to full length μ2 and an N-terminal truncation. The specificity of the yeast two-hybrid μ2/Yppϕ interaction was established by showing that mutant sequences of the YQRL motif, such as AQRL and YQRA, known to prevent TGN38 endocytosis, also fail to interact with μ2. Other motifs of this form, such as the YTRF sequence from the transferrin receptor, are also recognized by μ2.

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Specific interactions between peptides bearing the YQRL motif and either $\mu 2$ or purified AP-2 were also demonstrated in a number of biochemical experiments: for example, capture of in vitro translated $\mu 2$ to agarose beads coated with a YQRL-containing peptide, pull-down of intact AP-2 to agarose beads decorated with GST-fusions containing the YQRL motif, and surface plasmon resonance experiments designed to detect the association of AP-2 with the same GST-fusion constructs. Control experiments demonstrated that tyrosine is essential at the Y+0 position, and that amino acids with bulky hydrophobic resides are tolerated at Y+3. Similar experiments have now been performed with AP-1 (Boll et al 1996, Heilker et al 1996, Ohno et al 1996).

The use of the yeast two-hybrid system has been extended to the study of the interaction between μ-chains and the cytosoplasmic tails of a number of Yppϕ-containing proteins (Boge et al 1998, Bradshaw et al 1997, Shiratori et al 1997, Stephens & Banting 1998). These studies have provided the first, and at present the only, indication that Yppϕ motifs interact with the $\mu 3$ chain of AP-3 (Ohno et al 1998). It should be kept in mind that the associations detected by this method are qualitative and that it is difficult to assign affinities to these interactions without additional experimentation. A more direct method for identifying relevant interactions is to cross-link peptides containing the motif of interest and benzoyl phenylalanine (BPA) to intact APs. For functional Yppϕ motifs, UV illumination leads to specific chemically cross-linked adducts between the peptide and the $\beta 2$-subunit (Rapoport et al 1997). This method can also be used to identify factors that influence the ability of the AP complexes to recognize sorting signals (see below).

Specificity of Interaction All Yppϕ motifs are not equal. The intracellular traffic of Yppϕ-containing proteins is selective; rates of uptake into vesicles are different for different proteins, and some Yppϕ motifs bind far more poorly to AP-1 than to AP-2. For example, TGN38 and the transferrin receptor will pass directly through the TGN and reach the plasma membrane without being sorted to endosomes by AP-1-containing pits and vesicles, despite containing Yppϕ motifs that are fully functional for internalization to the endosome. Other Yppϕ motifs are more readily recognized by AP-1, such as the YQTI sorting motif found in the lysosomal protein lamp-1, which traffics from the TGN to the endosome.

The composition of residues at positions Y+1 and Y+2 appears to be particularly important for determining the intracellular site at which sorting occurs. Most strong endocytic signals, found in proteins with the highest endocytic rates, have a positively charged residue at Y+1 or Y+2; proteins that direct traffic from the TGN or from endosomes instead have a negatively charged or polar residue at these positions. This correlation has been verified by in vitro studies using surface plasmon resonance analysis, affinity chromatography peptide-AP UV-cross-linking, and yeast-two-hybrid interactions (Boll et al 1996, Ohno et al 1996, 1998), but the molecular basis for the preference is unknown.

Two complementary and powerful approaches have been used to examine the preferences of different μ chains for elements within the Yppϕ motif (Boll et al

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1996). Even when entirely unconstrained sequences are used, all three μ-chains select sequences of the form Yppϕ and selective binding depends primarily on the properties of the amino acids at positions Y+1 and Y+2. In one experiment, isolated recombinant GST-μ2 was screened for binding to members of an oriented combinatorial peptide library containing $\sim 10^9$ unique elements of the form MAXXXX**Y**XXXXAKKK (where X represents any of 16 amino acids; serine, threonine, tyrosine, cysteine, and tryptophan were excluded). Sequence analysis of the selected peptides showed a strong preference for arginine at Y+2 and leucine, methionine, or isoleucine at Y+3, consistent with the preference for large hydrophobic residues at this position. No detectable preferences were seen in the positions upstream and downstream of the motif, and it was concluded that motif recognition requires little in the way of context or tertiary structure. The second experiment, which led to similar conclusions, was based on the yeast two-hybrid system. Using a μ2 chain as the bait, a combinatorial library based on the cytosolic tail of TGN38 was constructed to serve as the prey; the YQRL motif was randomized to give a combinatorial library of $\sim 3 \times 10^6$ different sequences. In a modification of this experiment, the Y position was left alone, an amino acid with a bulky hydrophobic side chain (leucine, isoleucine, phenylalanine, methionine, valine) was placed at Y+3, and three residues upstream (Y-1, Y-2, Y-3), together with two residues downstream of it (Y+1, and Y+2), were randomized. This method was used to study the binding preferences of the μ1, μ2, or μ3 chains of AP-1, AP-2, and AP-3 (Ohno et al 1998). Some preference for sequences with proline at position Y+2 has been noted with this system, but the biological significance of this finding has not been established. The structure of part of the μ2 chain has now been determined in complex with 6-mer peptides containing either the YQRL or YRAL motifs (Owen & Evans 1998). Although these structures do not shed much light on the mechanism of selectivity, it is enlightening in a number of other ways (see below).

Is it significant that the Yppϕ motif is based on tyrosine? One can imagine that tyrosine phosphorylation of the motif could be used as a mechanism to regulate endocytosis, but no strong evidence for this has been observed. In vitro, tyrosine phosphorylation completely prevents recognition of the Yppϕ motif either by isolated μ chains or by intact APs (Boll et al 1996, Bradshaw et al 1997, Ohno et al 1996, Shiratori et al 1997). The only example so far known of a Yppϕ motif that is phosphorylated in vivo is the YVKM motif of CTLA-4, a negative signaling membrane protein receptor found in T cells (Walunas et al 1994, Krummel & Allison 1995) that interacts with AP-1 and AP-2 in vivo. This motif is used to retain CTLA-4 in the TGN and secretory vesicles and to direct its rapid internalization. Ionomycin-induced Ca^{2+} influx in T cells allows the rapid surface expression of CTLA-4, and this release in CTLA-4 retention is accompanied by phosphorylation of the YVKM motif (Alegre et al 1996, Linsley et al 1996). However, because the phosphorylated motif is used to recruit other SH2-domain-containing signaling molecules such as PI 3-kinase and SYP (Marengere et al 1996; Schneider et al 1995, 1999) to the cytoplasmic tail of CTLA-4, it is unclear whether the phosphorylation event also acts to control internalization.

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Atomic Structure of the μ2 Chain The structure of a fragment of the human μ2 chain (residues 122 to 435) has been determined, in two distinct co-crystals with peptides that correspond to the Yppϕ sorting motifs in EGFR or TGN38 (FYRALM and DYQRLN, for PDB entries 1BXX and 1BW8, respectively) (Owen & Evans 1998). The overall structure of μ2 is an elongated nine-strand β-sheet on the concave side of a banana-shaped domain of ~85 Å in length by ~30 Å in diameter (Figure 4, see color insert). The last strand is at the center and contains the C terminus of the polypeptide chain. Two smaller, four-strand sheets cover the convex surface of this nine-strand sheet (the banana domain). The residues that link the third and fourth strands, the fifth and the sixth strands, and the most N-terminal portion, are disordered. The extensive sequence similarity among the μ-chains implies that the structure of μ1 and μ3 will be essentially the same. The central position of the C-terminal strand suggests that the C-terminal deletions of μ1 in the *C. elegans* Unc-101 mutants (Lee et al 1994) would destabilize the entire fold, and this effect is probably the basis of the phenotype.

The site responsible for interaction of μ2 with the Yppϕ motif is on one side of the banana domain, between the concave and convex surfaces in the lobe closer to the N terminus (Figure 4). The motif binds across the edge of the large sheet. The phenol ring in the Y of the Yppϕ motif makes typical stabilizing contacts with the side chains of Phe_{174}, Asp_{176}, Lys_{203}, Trp_{421}, and Arg_{423}. The L at position Y+3 of both peptides is located in a hydrophobic pocket contributed by the side chains of Leu_{173}, Leu_{175}, Val_{401}, Leu_{404}, Val_{422} and the aliphatic portion of Lys_{420}. This pocket could accommodate hydrophobic residues other than L, consistent with the observation that I, M, and V sometimes appear instead of L in Yppϕ sorting signals. Comparison of the amino acids that vary among the μ-chains in the site that binds to Yppϕ shows modest differences suggesting a common mechanism of sorting signal recognition. The structure of μ2 does not, however, provide an explanation for the selective recognition of different Yppϕ sorting motifs by μ1, μ2, and μ3 chains based on the identity of the residues found at Y+1 and Y+2 because the side chains of these residues project into the solvent and do not make contacts with μ2.

The way μ2 recognizes tyrosine-containing peptides in μ2 is reminiscent of the recognition of phosphotyrosine-containing peptides by SH2 domains (Waksman et al 1992). Although the tyrosine must be phosphorylated for SH2 recognition and must not be phosphorylated for μ2 recognition, the residues contacting the tyrosine show similar geometry. In μ2 the pocket that accepts the phenolic hydroxyl OH is too small to accommodate a PO_3 group, and in SH2 domains the pocket is too large (and too positively charged) to accept an OH. The bulky hydrophobic residue that serves as the second anchor point in both motifs (at Y+3) inserts in a hydrophobic pocket on the opposite side of a β-sheet, and the backbone amides along the intervening sequence of the motif make contact with backbone carbonyls on the exposed edge of the sheet. The strategies that these two domains use to recognize a range of peptides with moderate selectivity are thus very similar, although the folds of the proteins are completely different. In the case of SH2 domains, however, the

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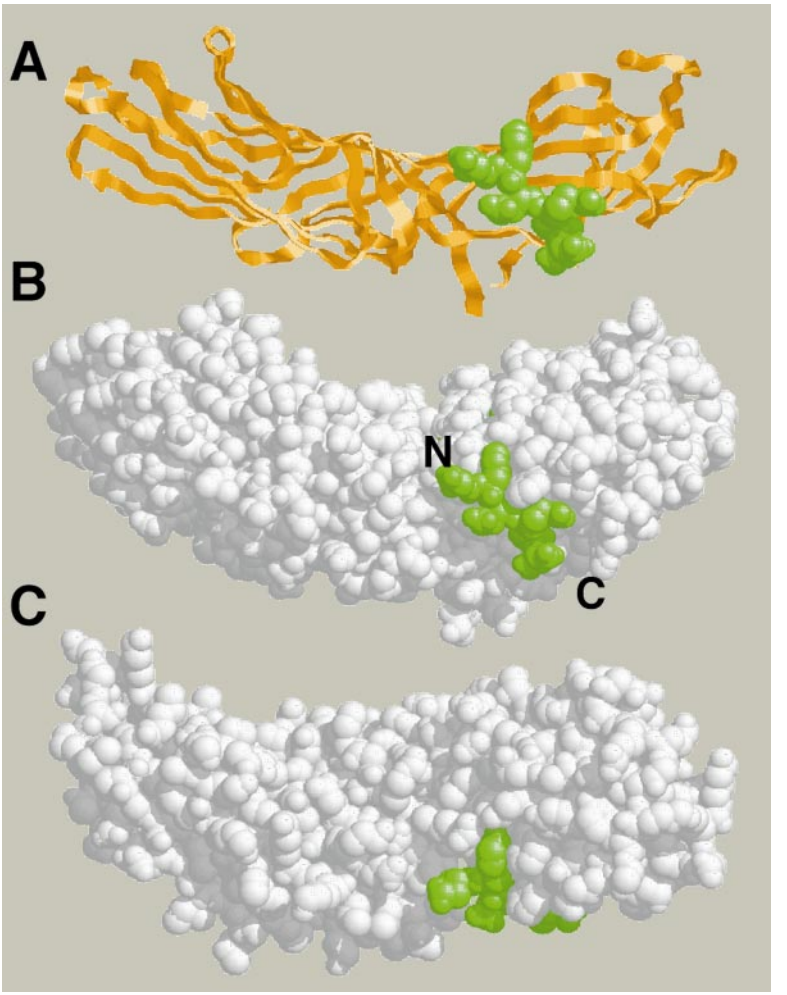

Figure 4 Atomic structure of the N-terminal fragment of the $\mu 2$ chain of AP-2 complexed with the peptide DYQRLN containing the Yppø-sorting motif of TGN38 (green). The representations in (*A*) and (*B*) correspond to bottom views, and (*C*) corresponds to a side view rotated about 90° from (*A*) and (*B*).

range of peptides bound is rather small, and there is little overlap between the classes of peptide bound by different SH2 domains. In the case of the μ chains the range of peptides bound is much larger, but it is still probably the case that there is little overlap between the peptides that bind well to $\mu 2$ and those that bind well to $\mu 1$ or $\mu 3$.

Because the μ chains within AP complexes are fairly resistant to cleavage (Matsui & Kirchhausen 1990, Schroder & Ungewickell 1991, Zaremba & Keen 1985), it is likely that the disordered portions of the μ chain make close contacts with other chains within the AP complex, constraining their conformation. When AP-2 complexes form clathrin coats, however, $\mu 2$ is efficiently cleaved at residues in the region between residues 210 and 240 in the disordered loop of $\mu 2$ (which lies close to the binding pocket for the Yppϕ motif) and at Lys_{281} (Matsui & Kirchhausen 1990) (also close to the binding pocket), suggesting that the disorder has a functional basis; the loop presumably adopts a new conformation when AP-2 binds to clathrin. Our group (Rapoport et al) has previously shown that AP-2 in coats has a higher affinity for Yppϕ motifs than free AP-2 (Rapoport et al 1997), indicating that the conformational change may expose the Yppϕ binding site.

Where are the μ chains in the AP complex? They are located in the 70–90 Å AP core (Zaremba & Keen 1985), together with the σ chain and ~600 residues of the N terminus of the two large chains (Kirchhausen et al 1989). The length of the partial μ chain is ~85 Å (Owen & Evans 1998), similar to the overall dimension of the AP core, which can be viewed as a rough cube or brick with sides ~60–80 Å in length. The overall dimensions of the μ chain are thus not much help in guessing its orientation. However, the peptide-binding site provides some clues. The end of the binding site that binds to the N terminus of the peptide is approximately 50 Å from one end of the banana domain and ~35 Å from the other end (Figure 5, see color insert). If the μ-chain is oriented perpendicular to the membrane, the N-terminal end (Y) of the Yppϕ sequence must reach at least 35 Å away from the membrane surface to contact the binding site. Yppϕ sequences are never closer to the membrane than 6 to 7 amino acids, and if the spacer is shortened, the motif becomes nonfunctional (Rohrer et al 1996), but a spacer of this length would stretch ~25 Å at most. Furthermore, the tail would have to buckle back on itself sharply to achieve the orientation required by the binding site. We therefore surmise that the μ-chain is oriented parallel to the membrane, and that the Yppϕ-containing cytoplasmic tail also lies parallel to the membrane, allowing binding without major distortions or unreasonable extension. These considerations also rule out certain models of μ's position within the core domain, for example, orientations that would position the peptide-binding site too close to the ears, where clathrin binding occurs (Dell'Angelica et al 1998, Gallusser & Kirchhausen 1993, Shih et al 1995, Traub et al 1995).

The LL Motif The dileucine, or LL, motif takes the form (−)(2-4)xLL, where x is usually a polar residue and (−) is often a negatively charged residue (aspartic

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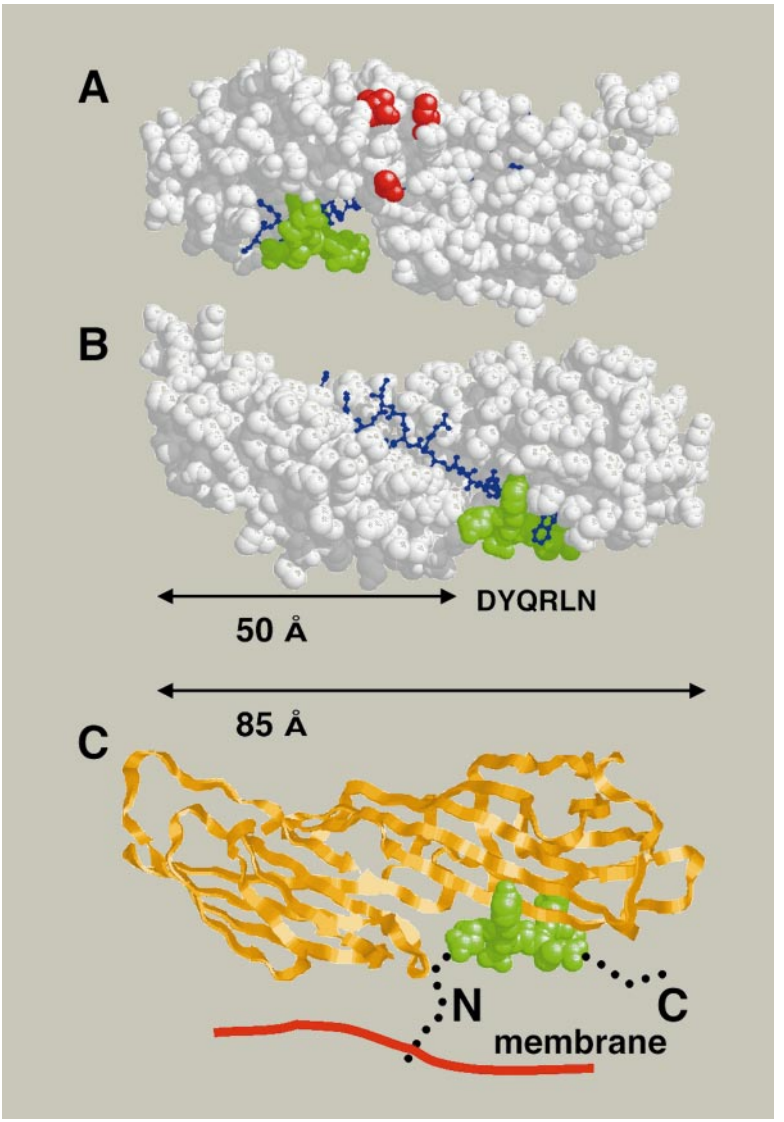

Figure 5 Atomic structure of the N-terminal fragment of the $\mu 2$ complexed with the peptide DYQRLN containing the Yppø-sorting motif of TGN38 (green). The hypothetical orientation of the fragment with respect to the membrane is based on the considerations provided in the text. The areas of proteolytic cleavage sites that become accessible in $\mu 2$ when AP-2 binds clathrin and forms coats are indicated (red). The β-strand affected by the C-terminal mutants of Unc 101 is shown as a ball and stock (blue). The side view in (*A*) has been rotated approximately 180° with respect to the vieres in (*B*) and (*C*). The vieres in (*B*) and (*C*) are equivalents.

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or glutamic acid or phosphoserine). At present, the LL motif is the only sorting signal other than the tyrosine motif known to be directly recognized by AP complexes. It controls the traffic of a variety of proteins through both endocytic and secretory pathways. LL motifs with the acidic residue are constitutively active, whereas those with serine are regulated by phosphorylation (see, for example, Geisler et al 1998). Unlike the Yppϕ motif, the LL motif is clearly context dependent: It is characteristically surrounded by polar and/or charged residues, but there is no obvious requirement for a particular sequence. This lack of sequence conservation has led to the suggestion that the main role of the polar/charged surrounding residues is to ensure that the hydrophobic leucine doublet is exposed to solvent. This notion received support from the observation that Nef, an essential HIV protein, uses the clathrin-dependent pathway to traffic to the endosomal compartment (Greenberg et al 1997, Mangasarian et al 1997). Its traffic depends on a LL motif at the C-terminal end of the protein (Bresnahan et al 1998, Craig et al 1998, Greenberg et al 1998). The structure of Nef shows that this portion of the molecule is a disordered surface loop (Grzesiek et al 1996, Lee et al 1996). Nef is the only nontransmembrane protein known to traffic using a LL motif; it is a cytosolic protein linked to the membrane by an N-terminal myristoyl group.

The LL motif was first identified in the cytosolic tail of CD3-γ, a transmembrane subunit of the T-cell receptor (Letourneur & Klausner 1992). Since then, it has been detected in a large number of trafficking proteins, including the mannose 6-P receptor, Limp-II, tyrosinase, Glut-4 (glucose) transporter, CD4 (the co-receptor for HIV), and Nef. Some membrane proteins carry more than one LL motif, and in some cases one of the leucines is replaced with either isoleucine or methionine. For example, the invariant chain of MHC class II molecules (Ii) contains two LL motifs, one ML and one LL. The effect, if any, of these variations on the rate and route of traffic is not yet understood.

The β-Chains Recognize the LL Motif The AP complexes bind directly to the LL motif in in vitro experiments using the cross-linking and surface plasmon resonance methods described above for the Yppϕ motif (Greenberg et al 1998, Honing et al 1998, Rapoport et al 1998). The results of phage display assay suggest that the N terminus of the μ-chain binds to the LL motif of invariant chain (Bremnes et al 1998), but the generality of this recognition has not been established. BPA-labeled peptides containing the LL motif cross-link selectively to the β1 and β2 chains in AP-1 and AP-2 complexes and not to the other subunits of the adaptor complex (Rapoport et al 1998). Consistent with this result, Yppϕ motifs (which bind to the μ chain) do not compete with LL motifs for binding to AP-1 or AP-2 in vitro, and saturation of the uptake mechanism for Yppϕ-containing proteins does not affect the uptake of LL-containing proteins in vivo (Marks et al 1996).

There are some indications that different AP complexes prefer different contexts for the LL motif: Surface plasmon resonance experiments have shown that mammalian AP-1or AP-2 (delivered in cytosolic extract) binds poorly to LL-containing peptides (containing the sequence DERAPLI) from LIMP-II or to LL-containing

peptides (containing the sequence DDYHSLL) from tyrosinase (Honing et al 1998), whereas the preference of AP-3 for the same peptides is reversed. Under the conditions used in this experiment, the dissociation constant appears to be in the high nanomolar range, although attempts to co-immunoprecipitate APs and LL-motif-containing proteins have consistently failed. The concentrations required for competition in the BPA cross-linking experiment are consistent with a K_d in the high micromolar range at best (Rapoport et al 1998). It is hard to resolve these apparently contradictory observations. APs have a tendency to aggregate (Beck & Keen 1991b), and it is therefore possible that the apparently strong interaction is the result of a multi-valent interaction between dimers, trimers or larger oligomers of APs and the immobilized peptide. It is also possible that the interaction between APs and the sorted proteins is strong but transient, causing co-immunoprecipitation to fail.

The importance of the LL motif in classical clathrin-dependent traffic—that is, the AP-1- and AP-2 dependent TGN-to-endosome and plasma-membrane-to-endosome pathways—has been known for some years (Letourneur & Klausner 1992). The recent discovery of AP-3 (Cowles et al 1997, Dell'Angelica et al 1997, Panek et al 1997, Simpson et al 1997, Stepp et al 1997) has led to a flurry of experiments to determine how closely the biological functions of AP-3 resemble those of AP-1 and AP-2. So far, it has been possible to determine that only AP-3 is indeed involved in the traffic of LL-motif-containing proteins (and also in the traffic of some proteins containing Yp$p\phi$ motifs). In yeast cells, the vesicular traffic of the membrane-bound alkaline phosphatase (ALP) from the Golgi to the vacuole requires both a functional AP-3 and a LV motif in the cytosolic tail of ALP (Stepp et al 1997, Vowels & Payne 1998). Similarly, the intracellular targeting of the yeast Vamp3 t-SNARE requires AP-3 and is based on a LL-sorting signal (Cowles et al 1997). Functional knockout by gene disruption of AP-3 subunits in mammals (Dell'Angelica et al 1999b, Kantheti et al 1998) leads to mistargeting of several proteins, including tyrosinase, that contain a LL motif sorting signal (Simmen et al 1999), and of the synaptic vesicle Zn (ZnT-3) transporter and the lysosomal membrane proteins CD63, lamp-1, and lamp-2, which contain Yp$p\phi$ motifs.

REGULATORY INPUTS

β-Arrestins

Is the sorting machinery regulated or constitutive? There are several points in the process of vesicle assembly and transport that appear to be under the control of molecular switches, coordinating the compartmentalization of vesicle coat components and cargo, and the timing of vesicle budding or release (for recent review, see Schmid 1997). The control of the internalization of activated β2-adrenergic receptors by β-arrestin1 and -arrestin2 (Goodman et al 1996) is a good example of the integration of multiple regulatory inputs to coordinate the uptake/sorting pathway.

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Three regulation points have been identified: control of β-arrestin transport to the membrane by phosphoinositides; control of β-arrestin binding to clathrin by phosphorylation; and control of β-arrestin binding to the receptor by receptor activation (see above). Mutated β-arrestin2 that lacks the ability to bind to phosphoinositides fails to localize to clathrin coated pits and to internalize activated receptors, even though it retains its ability to interact with clathrin and receptors, because it is unable to localize to the membrane (Gaidarov et al 1999). Most cytosolic β-arrestin1 is phosphorylated at Ser_{412} (situated in the C side of the clathrin-box) and must be dephosphorylated by an unknown phosphatase to bind to clathrin in vitro and to coated pits in vivo (Lin et al 1997). At least for β-arrestin1, control of membrane binding and control of clathrin binding appear to be coordinated. β-arrestin2 lacks this serine, however, and it is not clear whether its binding to clathrin is similarly controlled. Perhaps the relevant phosphatase is also membrane-bound.

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APs

There are also several possible ways to regulate the functions of the AP complexes, but in no case is the physiological significance of the regulation understood. There are significant differences between AP-1 and AP-2, and nothing is known about the regulation of AP-3 and AP-4.

The first hint that the function of APs could be regulated came from the observation that binding of phosphorylated inositols or phosphoinositides to the α chain in AP-2 complexes prevents the aggregation of AP-2 in vitro and inhibits its clathrin coat-assembly activity (Beck & Keen 1991a,b). It is possible that the binding site for these regulatory molecules, located in the α chain (Gaidarov et al 1996), is required for targeting of AP-2s to the plasma membrane. No equivalent inositol-binding activity has been found for the γ chain of AP-1, however. A second and distinct site for phosphoinositide binding is found in both AP-1 and AP-2. Recognition of Yppϕ sorting signals by AP-1 or by AP-2 is significantly enhanced by the presence of phosphoinositides that are phosphorylated at the D-3 position in the inositol ring (Rapoport et al 1997); the same phosphorylated lipids diminish the recognition of LL motifs (Rapoport et al 1998). Thus the presence of this lipid stimulus skews the transport machinery toward collection of Yppϕ-containing cargo and away from LL-containing cargo. The interaction between AP-2 and clathrin during coat formation also increases the affinity of AP-2 for Yppϕ motifs (Rapoport et al 1997). This effect is specific for Yppϕ, is not synergistic with the stimulation elicited by phosphoinositides, and does not influence the recognition of LL motifs. Again, AP-1 does not appear to be regulated in this way.

The interaction between APs and clathrin is almost certain to be controlled because empty cages are never observed in vivo. Cytosolic and, to a lesser extent, membrane-bound AP-1 and AP-2 adaptors are phosphorylated, particularly in several serine residues located close to the clathrin-binding box in the β-hinge

region (Wilde & Brodsky 1996). These phosphorylations prevent binding of APs to clathrin cages preformed in vitro. The identity of the phosphatases and kinases responsible for this regulation, their location, and the mechanisms by which their activity is in turn regulated, remain to be determined.

CLATHRIN PARTNERS

AP180 and CALM

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AP180 (also named NP185, F1-20 or AP-3, but not any longer in use) was first isolated from clathrin-coated vesicles purified from brains of cows, rats, or pigs (Ahle & Ungewickell 1986, Murphy et al 1991, Zhou et al 1993). Although the precise function of AP180 is not known, its abundance in brain tissues and the existence of the related protein CALM, which is ubiquitously expressed in other tissues, points to an important role in vesicular traffic (Dreyling et al 1996). Purified AP180 behaves as a monomeric protein and has a clathrin-binding domain (Morris et al 1993, Ye & Lafer 1995b) that contains at least one LLEFD clathrin-box. Purified AP180 efficiently drives clathrin assembly in vitro, showing the highest known coat assembly activity (Ahle & Ungewickell 1986, Lindner & Ungewickell 1992, Norris et al 1995, Ye & Lafer 1995a), and remains associated with the assembled clathrin coats. A region close to the N terminus of AP180 also binds to phosphoinositol lipids (Hao et al 1997, Norris et al 1995, Ye et al 1995). It is possible that this allows a signaling event, such as phosphorylation of a particular position on the inositol ring, to drive recruitment of AP180 to the membrane. However, it is as yet unclear the specificity by which the various phosphoinositol lipids can bind to AP180 in cells, and whether these lipids are present in sufficient abundance to explain recruitment.

AP180 also binds selectively to the plasma membrane AP-2 clathrin adaptor complex (described below) and not to the TGN-specific AP-1 adaptor (E Lafer, personal communication). This might indicate that plasma-membrane-derived coated vesicles would contain both AP180 and AP-2. It is also possible, however, that this interaction is an in vitro artifact or that three different populations of vesicles (containing AP180 only, AP-2 only, or a mixture) exist. Some insight into this question has resulted from the disruption of LAP, the homolog of the AP180 gene in *Drosophila* (Zhang et al 1998). In nerve terminals from normal tissue, the vesicles internalized in the pre-synapse in response to nerve-terminal stimulation are similar in size to the small AP180/clathrin coats formed in vitro. The synaptic vesicles are also about the same size and have been postulated to arise directly from the removal of the clathrin coat of the coated vesicles. In the mutant nerve terminals, the vesicles are significantly larger, similar in size to the coated vesicles found in nonbrain tissues, which contain AP-2 (and possibly CALM). It is thus possible that LAP (AP180) induces coat formation directly in nerve terminals, giving vesicles that have the characteristic size of AP180 clathrin coats formed in vitro. It is also possible that LAP (AP180) and AP-2 collaborate to form these vesicles, but

that LAP (AP180) dominates in forming the geometry of the coat. Nevertheless, LAP is insufficient simply to drive coat formation or to regulate only the size of the coats since mutations in *Drosophila* of the α-subunit of AP-2 (D-α*Ada*) completely block endocytosis (Gonzalez-Gaitan & Jackle 1997). At present there is no evidence that AP180 (or its relative CALM) interacts with the canonical sorting signals found in the cytosolic tails of cargo proteins. Thus one possible function of AP180 in nerve terminals could be to drive the rapid formation of coated vesicles without selecting cargo, effectively acting as a simple coat assembly adaptor to facilitate a relatively nonspecific recycling of plasma membrane.

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Other Partners

Although the problems of recognition, specificity, and regulation of cargo/AP/clathrin interactions are not solved, there has been considerable progress in all three areas in the last five years. Two sets of questions remain hard to address. How are the different AP complexes specifically targeted to their appropriate membrane? And why are the AP complexes so large?

A number of experiments have been performed with perforated cells showing that purified APs associate with their correct target membranes in a GTP and/or ATP-dependent manner (Robinson & Kreis 1992). So far, all that is known about the proteins responsible for the recruitment of AP-1 to TGN and endosomal membranes is that an activated GTP-ARF is required, although it has not been possible to demonstrate direct contacts between activated ARF and APs (Stamnes & Rothman 1993, Traub et al 1993, Wong & Brodsky 1992, Zhu et al 1998, 1999). SDS-PAGE has been used to show that a set of membrane-bound polypeptide bands, of unknown identity and function, bind to AP-1 in a GTPγS-dependent fashion (Mallet & Brodsky 1996, Seaman et al 1996). For AP-2, the situation is little different. Synaptotagmin, a membrane-bound protein found in synaptic vesicles and coated vesicles from brain that binds to AP-2, has been suggested as a candidate plasma membrane receptor for AP-2 (Zhang et al 1994). But synaptotagmin binds to the ear domain of the α chain of AP-2, and chimeras of AP-2 whose α-ear has been exchanged with the corresponding γ-ear of AP-1 continue to be targeted normally to the plasma membrane in live cells (Robinson 1993). Moreover, removal of the adaptor ears by limited proteolysis does not interfere with the recruitment of the remaining AP heads to the appropriate membrane (Peeler et al 1993, Traub et al 1995). It is therefore unlikely that synaptotagmin determines AP-2 plasma membrane targeting.

APs seem to be much larger than they need to be. The functions of APs identified thus far are their interaction with two different sorting signals, and an interaction with clathrin. Recognition of the Yppϕ motif requires a small patch on the surface of the μ chain, and the binding site for clathrin uses at most 30, and probably only 4 to 5, amino acids of the β chain. The sites responsible for binding to the LL motif, regulation of cargo recognition, and regulation of clathrin binding are probably similar in size. What is the rest of the 300-kDa complex doing? Some of

the possible functions for the "orphan" domains of APs include the control of dynamin recruitment to induce vesicle budding (Wang et al 1995), the control of transport to the vesicle's destination, and the recognition of the appropriate SNARE for fusion (Cowles et al 1997, Salem et al 1998). We will likely not know why APs are so large until we understand the functions of the increasing number of proteins that interact with them. For example, a variety of proteins have been found to interact with the ear domain of the α chain of AP-2, including Eps15, epsin, amphyphysin (all of unknown function), and perhaps dynamin (Benmerah et al 1996; Chen et al 1998, 1999; Iannolo et al 1997; Ramjaun & McPherson 1998; Wang et al 1995). The ear domain of the γ chain of AP-1 has been studied less, with only one partner, γ-synergin, definitively identified (Sowerby & Robinson 1996). Ataxia telangiectasia mutated (ATM) is another example of a protein that binds to APs, in this case to the β chain (Lim et al 1998).

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ARE APs REALLY NECESSARY FOR COAT FORMATION?

The accepted view is that AP-1 and AP-2 adaptors are essential for clathrin coat formation. The original concept arose from the observation that AP-1 and AP-2 can interact with clathrin and drive the assembly of clathrin coats in vitro, under physiological buffer conditions that prevent pure clathrin from forming coats (Keen 1987, Keen et al 1979, Pearse & Robinson 1984, Zaremba & Keen 1983). The idea was bolstered by the observation that in cells most clathrin-coated membranes contain AP-1 or AP-2; together, these two facts led to the assumption that APs are essential for coated-pit and coated-vesicle formation. Experiments with mammalian cells designed to interfere with the function of AP-2 appear to confirm this assumption; both injection of antibodies specific for AP-2 into intact cells (Chin et al 1989) and immuno-depletion of AP-2s in permeabilized cells prevent endocytosis (Smythe et al 1992). Moreover, overexpression in mammalian cells of known partners of AP-2 could inhibit endocytosis (Benmerah et al 1998).

The first challenge to the dogma, that AP-1 and AP-2 adaptors are essential for coat assembly came when a selective knockout of the yeast APl2 gene, which encodes the β chain of the AP-1/AP-2 adaptor, showed an unexpectedly normal phenotype in cells expressing normal clathrin (Rad et al 1995). The phenotype for genetic disruption of clathrin function, in contrast, is significant: the cells grow extremely slowly and show characteristic defects in vesicular post-Golgi traffic. If APs are required for clathrin assembly, the two phenotypes should be similar. There are, however, two other AP complexes in yeast, and it was easy to assume that these were compensating for the removal of the AP-l and -2 function.

Recently it was discovered that simultaneous disruption of all known AP subunits not only fails to generate the clathrin ($^-$) phenotype but also has no noticeable effect on the generation of clathrin-coated vesicles in the mutant yeast cells (Huang et al 1999: G Payne, personal communication). Moreover, the additional disruption of the two genes for yeast proteins homologous to AP180/CALM still fails

to affect the formation of clathrin-coated vesicles or to produce a dramatic phenotype (Huang et al 1999). These results raise the possibility that other proteins remain to be discovered that, at least in yeast, can link clathrin to membrane-bound cargo and support coat assembly.

A similar genetic test for the dependence for coat assembly on AP function could in principle be performed in multicellular organisms. Thus far this approach has not been feasible: disruption of the γ chain of AP-1 in mice is embryonic lethal (Zizioli et al 1999); expression of strong mutant alleles of D-α*Ada*, the α chain of AP-2 in *Drosophila*, completely blocks endocytosis in pre-synapses (Gonzalez-Gaitan & Jackle 1997), and partial disruption of the functions of these proteins by the heterozygotic expression of mutant proteins leads to incomplete phenotypes that are hard to interpret in terms of the mechanism of coat assembly. Clearly, the AP complexes are more important for multicellular organisms than for yeast, but is this because a specific essential cargo molecule is being mis-trafficked, or because there is a general failure in clathrin coat assembly? A concerted effort to collect or construct individual cells that lack AP complexes will be necessary to answer this question.

A second frustration in understanding the true functions of AP-1 and AP-2 in multicellular organisms comes from the lethality of their genetic disruption; it is hard to characterize a lethal defect. Which of the cargo molecules is mislocalized as a result of AP-1 or AP-2 disruption, and which of these is responsible for the lethal phenotype? It is easier to study these questions for the AP-3 complex. Genetic disruption of AP-3 chains in *Drosophila* (Ooi et al 1997, Simpson et al 1997), mice (Kantheti et al 1998), and humans (Dell'Angelica et al 1999b) has shown that although the AP-3 function is not essential, it can result in significant sorting defects. Unfortunately this does not necessarily tell us much about the physiological functions of AP-1 and AP-2 because it is not clear whether there is a biologically significant association between clathrin and AP-3 (Dell'Angelica et al 1997, 1998; Simpson et al 1997).

CONCLUSIONS

The original hypothesis for the existence of a group of molecules that act as adaptors between the clathrin coat and the cytosolic tails of cargo proteins has been fulfilled and surpassed. It is now clear that several types of proteins have evolved to serve this function: Sometimes they are relatively simple monomeric proteins specialized to interact with a single type of cargo (the case of the β-arrestins) and sometimes they are more complicated structures that can recognize a number of different types of cargo (the case of the APs). The complexity of their functional regulation is becoming apparent, and often their control involves the coordinated action of lipids and proteins. It is now also evident that many of the interactions among components of the coated-vesicle assembly are of relatively low affinity, a characteristic that probably is needed to ensure rapid engagement and dissociation.

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It is also becoming apparent that several of the molecules that function in the clathrin pathway make more that one contact with each other; understanding the functional significance of this multivalency is an important challenge. Although we have a general picture of how sorting occurs, it is still hard to describe in detail how cargo is recruited, how the various adaptors are targeted to the correct membrane compartment, and how they disengage so that another cycle of coat assembly can occur. Solutions to these puzzles are likely to emerge in the next few years.

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