A Large-Scale Conformational Change Couples Membrane Recruitment to Cargo Binding in the AP2 Clathrin Adaptor Complex

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SUMMARY

The AP2 adaptor complex (α, β2, α2, and μ2 subunits) crosslinks the endocytic clathrin scaffold to PtdIns4,5P2-containing membranes and transmembrane protein cargo. In the “locked” cytosolic form, AP2’s binding sites for the two endocytic motifs, YxxΦ on the C-terminal domain of μ2 (C-μ2) and [ED]xx[L][L] on α2, are blocked by parts of β2. Using protein crystallography, we show that AP2 undergoes a large conformational change in which C-μ2 relocates to an orthogonal face of the complex, simultaneously unblocking both cargo-binding sites; the previously unstructured μ2 linker becomes helical and binds back onto the complex. This structural rearrangement results in AP2’s four PtdIns4,5P2- and two endocytic motif-binding sites becoming coplanar, facilitating their simultaneous interaction with PtdIns4,5P2/cargo-containing membranes. Using a range of biophysical techniques, we show that the endocytic cargo binding of AP2 is driven by its interaction with PtdIns4,5P2-containing membranes.

INTRODUCTION

The regulated movement of proteins and lipids between the many cellular membranes in coated vesicular carriers is important for signaling, homeostasis, defining the interactions of cells with their surroundings, and in controlling the glycosylation and proteolytic processing of luminal and transmembrane proteins. Clathrin-coated vesicles (CCVs) mediate many post-Golgi trafficking routes including internalization from the plasma membrane via clathrin-mediated endocytosis (CME) (Traub, 2009). CCVs have a three-layered structure with an inner membrane layer linked by clathrin adaptors to an outer polymeric clathrin scaffold (Cheng et al., 2007; Fotin et al., 2004). Clathrin adaptors contain a folded membrane-proximal domain, which binds to phosphatidylinositol polyphosphate (PIP) headgroups and/or Arf GTPases in their membrane-attached, GTP-bound forms, and at least one natively unstructured region, which harbors a clathrin-binding motif (Owen et al., 2004). Transmembrane proteins are generally selected as cargo for incorporation into a CCV through the direct interaction of either widely used, short, linear sequence motifs or covalently attached ubiquitin chains with the membrane-proximal portion of a clathrin adaptor (reviewed in Bonifacino and Traub, 2003; Traub, 2009).

The family of heterotrimeric vesicle coat adaptors comprises APs 1–4 and the β, γ, δ, ε subcomplex of COPI (Schiedewski et al., 1999). AP2 consists of α, β2, μ2, and α2 subunits (Figure S1 available online) and is the most abundant endocytic clathrin adaptor (Keen et al., 1981; Pearse and Robinson, 1984). The 70 kDa trunk domains of α and β2, together with the 50 kDa μ2 and the 17 kDa α2 subunits, form the 200 kDa membrane-proximal core (Collins et al., 2002; Heuser and Keen, 1988). The 30 kDa bilobal C-terminal appendages of α and β2 each possess two sites for binding different motifs found on many other endocytic CCV coat proteins with various accessory/regulatory functions in CCV formation (reviewed in Owen et al., 2004; Schmid and McMahon, 2007). The appendages are connected to the core via long flexible linkers (Zaremba and Keen, 1983) that contain clathrin-binding motifs. The AP2 core is the site of binding to the two widely used endocytic cargo motifs, YxxΦ (where Φ is a hydrophobic residue: I, L, M, F, or V) and [ED]xx[L][L] or “acidic dileucine” motifs, as well as to the plasma membrane PIP, phosphatidyl inositol-4,5-bisphosphate (PtdIns4,5P2). The AP2 complex therefore acts as a central hub for CCV formation, binding to clathrin, cargo molecules, accessory proteins, and membranes, and these interactions must be under strict spatiotemporal control.

Two structures of the AP2 core have been solved, one with a PIP headgroup analog (inositol hexakisphosphate [IP6]) but
lacking bound Yxx\(\Phi\) or [ED]xxx[LI] motifs (Collins et al., 2002), and the other in complex with an [ED]xxx[LI] motif peptide (Kelly et al., 2008). The \(\alpha\) and \(\beta\) trunk domains are solenoids of stacked \(\alpha\) helices, and the N-terminal \(\mu\) domain (N-\(\mu\); residues 1–135) and \(\beta\) domain are globular “longin domain” folds. Together, these four domains are arranged into a “bowl,” in which the elongated C-terminal \(\mu\) domain (C-\(\mu\)) sits (Figure S1).

Two positively charged sites that can bind PtdIns4,5P2, one on \(\alpha\) and one on \(\beta\), have been identified (Collins et al., 2002; Gaidarov et al., 1996; Rohde et al., 2002) with the basic site on \(\alpha\) playing a key role in the initial docking of AP2 onto PtdIns4,5P2 and cargo-containing membranes (Gaidarov et al., 1996; Höning et al., 2005).

The binding site for [ED]xxx[LI] motifs is located mainly on \(\alpha2\) (Chaudhuri et al., 2009; Doray et al., 2007; Kelly et al., 2008), whereas Yxx\(\Phi\) motifs bind through a \(\beta\)-augmentation to C-\(\mu2\) (Ohno et al., 1995; Owen and Evans, 1998). In the motif-free conformation first shown for AP2 and subsequently for AP1 (Heldwein et al., 2004), both cargo-motif binding sites are blocked by portions of the \(\beta2\) subunit: \(\beta2\)Tyr6 and \(\beta2\)Phe7 block the [ED]xxx[LI]-binding site and \(\beta2\)Glu364–\(\beta2\)Val406 (especially \(\beta2\)Val365 and \(\beta2\)Tyr405) block the Yxx\(\Phi\) site (numbering refers to AP2) (Figure S1), and thus we term this the “locked” conformation. In the recent [ED]xxx[LI] motif-ligated structure, which we term the “unlatched” conformation, the N terminus of \(\beta2\) is displaced, allowing [ED]xxx[LI] motif binding to occur. Although in the unlatched form the subunits of the bowl have moved a little relative to each other as compared with the locked form, the Yxx\(\Phi\) motif-binding site remains blocked (Kelly et al., 2008). In both the locked and unlatched structures, the main PtdIns4,5P2-binding site on the \(\alpha\) subunit and the [ED]xxx[LI] motif-binding site are adjacent to each other and located on what was consequently proposed to be part of the AP2 membrane-interacting surface, but the Yxx\(\Phi\) motif-binding site is located on an orthogonal face. It has also been shown that the spacer between the end of a protein’s transmembrane helix and a Yxx\(\Phi\) motif need only be seven residues (around 25 Å-bound conformation, AP2 core was preincubated with a very large (70-fold) molar excess of the TGN38 Yxx\(\Phi\) peptide DYQRLN (35 \(\mu\)M AP2 with 2.5 mM peptide). Crystals of the AP2 complex with bound peptide grew under a number of conditions, but only those grown using mixtures of ammonium sulfate and lithium sulfate showed useful diffraction. These rhombohedral crystals diffracted anisotropically to 3.1 Å resolution in the best direction but little beyond 5 Å in the worst. Experimental phasing was carried out using the Ta\(_6\)Br\(_{12}\)\(^{2+}\) cluster compound and cryo-trapped Xe derivatives, followed by density modification. The bowl of the AP2 core and C-\(\mu2\) could be seen in the resulting electron density (Figures 1A and 1B). It was immediately obvious that a large conformational change had occurred, as C-\(\mu2\) was no longer located in the center of the bowl but on an orthogonal face (Figures 1E and 1F, compare Figures 1C and 1D).

Model building began by placing rigid-body components of the unlatched AP2 structure into the electron density. Electron density was visible for the linker connecting the two domains of \(\mu2\), showing that most of it adopted a helical conformation (Figure 2A), and for the Yxx\(\Phi\) peptide (Figure 2B). Surprisingly, electron density was also visible in the [ED]xxx[LI] motif-binding site on \(\alpha2\), although no appropriate exogenous ligand had been included in the crystallization experiments. Inspection of the electron density (Figure 2C) suggested that this “phantom” [ED]xxx[LI] motif was part of the myc-tag sequence (MEQKLI) inserted into a surface loop of the AP2 core lacking bound C-\(\mu2\) altogether (\(\mu2\)-truncated core) also adopts the myc-tag sequence (MEQKLI) inserted into a surface loop of \(\mu2\) (residues 218–252) at residue 236, given that at low contour levels almost continuous density could be seen extending from the dileucine-binding site to residue 252. This loop was disordered in the locked and unlatched forms. In this “open” form, the myc-tag-containing loop reaches over to a neighboring molecule and forms a vital crystal-packing contact. When the myc-tag was removed from the loop, the resulting protein yielded no diffracting crystals under any crystallization conditions including those used here.

### Conformational Change in the AP2 Bowl

The AP2 bowl can be considered as an extended helical solenoid running continuously from the N terminus of \(\alpha\) through the \(\beta\) subunits’ C termini to a dozen residues short of the N terminus of \(\beta2\), forming a puckered ring like the seam of a tennis ball. When going from locked to the open form, the AP2 bowl collapses inwards, expelling C-\(\mu2\) (Figure 3, Figure S2, and Movie S1 and Movie S2). The collapse brings both lobes of the puckered ring closer together, while the ring dislocates between the \(\alpha\) and \(\beta2\) N termini. The “collapsed” conformation of the bowl is likely to be its lower energy state as a version of the core lacking C-\(\mu2\) altogether (\(\mu2\)-truncated core) also adopts the collapsed conformation (see Extended Experimental Procedures).

The collapse of the AP2 bowl is facilitated by rotations about four “hinge points,” two each in \(\alpha\) and in \(\beta2\) (Figure S2). Thus each large subunit can be considered as being composed of essentially three rigid groups. The total buried subunit interfaces for the locked and open forms are 10,040 Å\(^2\) and 9700 Å\(^2\), respectively. Given that buried surface area is highly correlated with energy and therefore stability (Krissinel and Henrick, 2007), these data suggest that the locked form is more stable and therefore predominates in solution. The major contributions to the buried...
subunit interfaces come from the interactions between \( \alpha \) and \( \alpha' \), \( \alpha \) and \( \beta' \), \( \beta \) and \( \beta' \), and \( \beta' \) and \( N' \), which remain largely unaltered during the conformational change (see Table S1 and Figure S3), whereas the major changes in subunit packing are those made by \( C' \) and the N terminus of \( \beta' \) being displaced from the \( [ED]xxxL[L] \) motif-binding site on \( \alpha \) (Kelly et al., 2008).

Figure 1. Structure of the Open Conformation of the AP2 Core

(A) Part of the experimentally phased, solvent-flattened electron density map with the refined model superimposed. (B) The experimental electron density map in the region of the C-terminal domain of \( \mu' \), showing good density for the first subdomain (left) but very weak density for the poorly ordered second subdomain (right).

Membrane Binding and the Repositioning of \( C' \)

The most spectacular subunit rearrangement, and that of the greatest biological significance, is the movement of \( C' \) relative to the bowl. This can be described geometrically as a screw rotation of \( C' \) by \( \sim 129° \) about its long axis, with a translation of 39 Å (about half its length), relative to \( N' \) (Figure 4A). The trajectory followed by \( C' \) during the actual conformational switching must be greater than this in order to avoid colliding with \( \beta' \) (see Movie S3). The subunit contacts made by \( C' \) change completely between the locked and open conformers (Figure 4B). All contacts between \( C' \) and \( \alpha \) and between \( C' \) and \( \alpha' \) are lost, and although the surface area buried between \( C' \) and \( \beta' \) doubles (see below and Figure S3 and Table S1), the total subunit interface area buried by \( C' \) in the open form drops by around 1200 Å\(^2\) as compared with the locked form. However, this loss of buried surface area is partly compensated for by the 800 Å\(^2\) surface area buried by the \( \mu' \) linker binding back onto the rest of the core (see below, Figure 4 and Table S1).

In the locked and unlatched forms, the \( \mu' \) linker (residues 130–158), which contains the AAK1 (\( \alpha' \)-appendage binding kinase) catalyzed phosphorylation site at \( \mu' \)Thr156 (Conner and Schmid, 2002), is disordered, thus making it ideally suited to recognition by the protein kinase. This phosphorylation is important for AP2 function in vivo because mutating the phosphorylation site inhibits transferrin uptake (Motley et al., 2006; Semerdjieva et al., 2008). In vitro,\( \mu' \)Thr156 phosphorylation enhances the binding of AP2 to endocytic motifs (Höning et al., 2005; Olusanya et al., 2001; Ricotta et al., 2002) presumably by driving the equilibrium toward the cargo-binding-competent open form. In the open form the linker forms a four-turn helix, which packs in a trough lined by residues from \( \beta' \), \( N' \), and \( C' \) (Kelly et al., 2008). In subsequent pictures this myc loop is omitted for clarity, but the EQKLI sequence is shown in its position on \( \alpha' \).
of the linker by interacting with positively charged residues in the vicinity of the trough, especially \( \beta_2 \text{Arg138} \) and \( \beta_2 \text{Lys139} \) (Figure S3). Unfortunately all attempts to crystallize the phosphorylated form of AP2 have so far failed, and attempts to mutate candidate residues resulted in poorly expressed, aggregation-prone complexes. Further, an attempt to mimic the phosphorylation event by mutating \( \mu_2 \text{Thr156} \) to glutamate was also unsuccessful: \( \mu_2 \text{Thr156Glu} \) core shows 4-fold weaker binding to PtdIns4,5P2-containing liposomes than wild-type core and 20-fold less binding than phosphorylated AP2 core (S.H., unpublished data).

The result of the relocation of C-\( \mu_2 \) is that all three previously biochemically confirmed ligand-binding sites (those for PtdIns4,5P2, Yxx\( \Phi \) motifs, and [ED]xxxL[IL] motifs) become coplanar on a surface of AP2 and are thus suitably arranged for contacting the various signals in the context of the plasma membrane. There are three other regions of highly positive electrostatic potential on this surface in addition to the \( \alpha \) subunit PtdIns4,5P2-binding site (Figure 5). The first is formed by basic residues from the N terminus of \( \beta_2 \) (Lys5, Lys12, Lys26, Lys27, Lys29, Lys36). A version of the AP2 core in which these residues are mutated to glutamate (\( \mu_2 \text{PIP-Core} \)) cannot be recruited to PtdIns4,5P2-containing membranes, similar to the version of the AP2 core harboring mutations in the PtdIns4,5P2-binding site on \( \alpha \) (\( \alpha \text{PIP-Core} \)) (Figure 5 and Hönig et al., 2005). The second and third basic regions are on the surface of C-\( \mu_2 \). The second basic region (Lys330, Lys334, Lys350, Lys352, Lys354, Lys356, Lys365, Lys367, Lys368, Lys373) was identified as a putative PtdIns4,5P2-binding site (Collins et al., 2002; Rohde et al., 2002). Mutation of five lysine residues to glutamate in this region (5K\( \rightarrow \)E) slightly weakens the binding of AP2 cores to PtdIns4,5P2-containing liposomes (dissociation constant \( K_D \) 11 \( \mu \text{M} \) instead of 7 \( \mu \text{M} \)). In the third region (Lys167, Arg169, Arg170, Lys421), the substitution of three basic residues (KRR\( \rightarrow \)E) has little effect on AP2 binding to PtdIns4,5P2-containing membranes. However, combining the mutations (\( \mu_2 \text{PIP-Core} \)) results in an AP2 core with a 4-fold reduction in binding to PtdIns4,5P2-containing membranes. All mutant forms of the AP2 core were correctly folded as judged by identical levels of expression, incorporation into complexes, and CD spectra (data not shown). These data suggest that initial membrane recruitment of AP2 occurs mainly through the \( \alpha \) and \( \beta_2 \) PtdIns4,5P2-binding sites with the basic surface on C-\( \mu_2 \) playing an auxiliary role. However, we propose that the C-\( \mu_2 \) basic regions are key to driving the opening of AP2 by the electrostatic attraction of C-\( \mu_2 \) to the PtdIns4,5P2 membrane, as the \( \mu_2 \text{PIP-Core} \) mutant shows strongly inhibited (20-fold) binding to PtdIns4,5P2/Yxx\( \Phi \) liposomes. The binding of the \( \mu_2 \text{PIP-Core} \) mutant to PtdIns4,5P2/[ED]xxxL[IL] liposomes is only reduced by 3-fold, suggesting that acidic dileucine motif binding does

**Figure 2. New Structural Features Not Found in the “Locked” Structure**

The top panels are “omit” maps, mFo-DFc difference maps calculated by omitting part of the structure, randomly displacing all the atoms a little and then refining, using the experimental phases as restraints. Omitted residues are colored red (linker) or yellow (Yxx\( \Phi \) peptide and dileucine peptide mimic). The lower panels show the solvent-flattened experimental map.

(A) The \( \mu_2 \) linker folds into a helix lying in a groove between N-\( \mu_2 \) and \( \beta_2 \): the side chain of Thr156, which can be phosphorylated, is shown.

(B) The Yxx\( \Phi \) motif peptide is bound to the C-\( \mu_2 \) domain, in the equivalent position to that found on the isolated C-\( \mu_2 \) domain (Owen and Evans, 1998).

(C) Electron density in the acidic dileucine peptide-binding site on \( \alpha \) is linked to C-\( \mu_2 \) across a crystal contact and has been interpreted as part of the myc-tag EQKL. The peptide QIKRL from the unlated structure (gray) is shown in its position relative to \( \alpha \).
regions, and even in these cases (CIMPR [Chen et al., 1993], separated by at least 20 residues within their unstructured showed that in only a very few cases do both motifs occur membrane proteins (M. Robinson, personal communication) around 25 residues), but this cannot formally be demonstrated in our assay system. Inspection of a database of type I and II ture suggests that both motif-binding sites could be occupied simultaneously. We would therefore predict that AP2 would exhibit tighter binding to a membrane harboring both motifs than to one with either motif alone, due to the avidity effect of multiple PtdIns4,5P₂ molecules arranged in a roughly planar fashion in the membrane. This is supported by three lines of evidence. First, if no PtdIns4,5P₂ is included in liposomes that contain both Yxxφ and [ED]xxxL[LI] motifs, there is no detectable binding of AP2 (Figure 6). Second, mutation of any one of the basic PtdIns4,5P₂-binding patches on α or β₂, or the combined pair on μ₂, strongly inhibits binding to membranes containing PtdIns4,5P₂ and Yxxφ-sorting motifs (Figure 5 and Figure S4), i.e., high-affinity binding to Yxxφ-containing membranes requires multiple simultaneous PtdIns4,5P₂-binding events on α, β₂, and μ₂ that can only occur when AP2 is in its open conformation. Finally, in the fluorescence anisotropy assay measuring the equilibrium association of AP2 with fluorescein-labeled Yxxφ peptide, the binding of AP2 was strongly promoted by the presence of the polyanionic heparin (~50-mer), which, with multiple negative charges disposed along a single large molecule, can mimic the arrangement of charges in a membrane. The binding for AP2 to free Yxxφ peptide increases from undetectable levels to a K_D of ~3.4 μM in the presence of heparin, which is a similar strength of binding to that of isolated C-μ₂ for the same peptide, K_D ~1.9 μM (Figure S5).

To explore the nature of AP2 activation further we used polarized fluorescence stopped-flow spectrophotometry to follow the pre-steady-state kinetics. Isolated C-μ₂ and AP2 preincubated with heparin both display rapid binding to Yxxφ peptide (Figure S5), with fast on-rates (~10^6 M⁻¹ s⁻¹) and off-rates of ~2 s⁻¹. The kinetically derived dissociation constants of C-μ₂ and heparin-incubated AP2 (0.5 μM and 3.5 μM, respectively) closely match equilibrium measurements, confirming that interaction occurs in a single step. In contrast, Yxxφ binding by AP2 in the absence of heparin occurs extremely slowly (Figure S6). Heparin-activated AP2 binds with a relaxation time

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**Figure 3. The Conformational Change in AP2 on Cargo Binding**

Schematic (left) and surface (right) representations of the open (upper) and locked (lower) conformations. In the transition from the locked to the open structure, the puckered ring formed from α and β₂ narrows and splits between the N termini, and C-μ₂ emerges from its bowl and rotates roughly about its long axis. α₂ and N-μ₂ remain fixed to α and β₂, respectively.

Endocytic Motif Binding

Once attached to and stabilized on the membrane through binding multiple PtdIns4,5P₂ molecules, the open form of the complex can be further stabilized by the binding of membrane-embodied Yxxφ and [ED]xxxL[LI] cargo motifs in their respective sites, to which there is now unrestricted access for cargoes embedded in the membrane (Figure 6). Our motif-ligated structure suggests that both motif-binding sites could be occupied simultaneously. We would therefore predict that AP2 would exhibit tighter binding to a membrane harboring both endocytic motifs than to one with either motif alone, due to the avidity effect of simultaneous binding. This is indeed the case (Figure 5 and Figure S4). However, whether two different cargoes can bind simultaneously to a single AP2 in vivo will depend on steric clashes between their extracellular domains, the flexibility of their juxtamembrane regions, and the ratios of clathrin:AP2: cargo in a given CCV.

The open form structure suggests that a single cargo protein containing both motifs could, in principle, bind with both motifs engaged by the same AP2 complex, but only if the motifs were separated by at least 65–70 Å (corresponding to a spacing of around 25 residues), but this cannot formally be demonstrated in our assay system. Inspection of a database of type I and II membrane proteins (M. Robinson, personal communication) showed that in only a very few cases do both motifs occur separated by at least 20 residues within their unstructured regions, and even in these cases (CIMPR [Chen et al., 1993], furin [Voorhees et al., 1995], vGlut-1 [Kim and Ryan, 2009], and LRP1 [Li et al., 2001]), which have complex trafficking itineraries, it appears that one motif is used mainly for internalization and the other for intracellular trafficking.

A question that naturally arises is, if this open structure is energetically stable, is the locked, non-cargo-binding form biologically relevant, i.e., is it the conformation of AP2 in solution? This seems likely, as in the absence of ligands, the locked conformation buries a larger total surface area and is therefore more stable than the open form; furthermore, the locked conformation has also been seen independently in AP1 (Heldwein et al., 2004). Two further lines of evidence support the hypothesis that AP2 adopts the locked conformation in solution. First, in a surface plasmon resonance (SPR) assay, preincubation of AP2 cores with an excess of Yxxφ motif-containing peptide has no effect on the association of AP2 with PtdIns4,5P₂ and Yxxφ motif-containing liposomes, whereas preincubation of isolated C-μ₂ with an excess of the same peptide is sufficient to abolish binding to the same liposomes (Figure S5). Second, equilibrium fluorescence anisotropy experiments in which a fluorescein-labeled Yxxφ motif-containing peptide was titrated with C-μ₂ or with AP2 core reveal that isolated C-μ₂ binds the peptide with a K_D of ~1.9 μM, whereas the AP2 core exhibits no detectable binding (Figure S5).
(a measure of the time in which binding occurs) of 0.056 s, whereas in the absence of heparin this reaction slows 1000-fold to >60 s (Figure 6). The dramatic difference in kinetics can only be explained by a slow rate-determining reaction that precedes the bimolecular binding step. As the stoichiometry of the AP2:YxxF interaction is known to be 1:1, the preceding slow step must represent a pre-equilibrium isomerization to a binding-competent isomer. Calculation of kinetic rate constants (data not shown) gives a derived KD for AP2:YxxF of 4 mM. The difference between the affinities of AP2 in the presence or absence of heparin suggests that, at equilibrium, >99.9% of non-membrane-bound AP2 is in a locked or inactive conformation. These data show that AP2 is thus unable to associate promiscuously in the cytoplasm with proteins containing trafficking motifs.

DISCUSSION

The striking conformational change between the locked and the open, ligand-bound active forms of AP2 described here completely remolds the domain arrangement of the heterotrimer. Based on the known AP2 core structures, we propose the following scenario for the activation of AP2 for cargo binding on the plasma membrane. The model assumes that there are two main conformers of AP2, one locked and one open, that are in equilibrium with each other in solution, but that the equilibrium lies heavily in favor of the closed form in the absence of membrane interaction. By characterizing the pre-steady-state interaction of AP2 with a YxxF peptide, we have provided direct evidence that AP2 isomerizes between inactive (“locked”) and active (“open”) isomer forms and shown that >99.9% of non-membrane-bound AP2 in the cell will be in the “locked” conformation. The “activated” form of AP2 binds YxxF peptide with a rapid association rate similar to that of isolated C-m2. This is what we would expect from our structure because the YxxF-binding site on C-m2 is now unobstructed by any part of the complex. Taken together with the coplanar arrangement of all the ligand-binding sites, from which we would predict effects subsequently confirmed by our mutagenesis data (Figure 5), it seems reasonable to equate our open structure with the “activated” form of AP2 on the plasma membrane.

The first step in AP2 activation is its recruitment onto the plasma membrane, primarily by binding through the basic patches on α or β2 to PtdIns4,5P2, which in vitro has an apparent KD of 7–8 μM (Höning et al., 2005; Figure 5). Once attached to the membrane via α and β2, the electrostatic attraction and subsequent binding of C-m2 to the high local concentration of PtdIns4,5P2 causes AP2 to adopt the open conformation. The μ2 linker can now be buried in a helical conformation in the slot formed between N-m2 and β2, again stabilizing the open form. Further stabilization may be achieved through phosphorylation of μ2Thr156 by AAK1, resulting in the apparently tighter binding of μ2Thr156-phosphorylated AP2 to cargo-containing membranes (Höning et al., 2005; Ricotta et al., 2002).
The initial dislocation of C-μ2 from its site on the bowl causes the bowl to relax toward the lower-energy conformation present in the open and μ2-truncated forms of the AP2 core (Extended Experimental Procedures). The most important functional effect of this change in the conformation of the bowl is to cause the β2 subunit to move with respect to the two peptide ligand-binding sites such that it is no longer able to block either. The β2 subunit can therefore be considered as the latch that in solution blocks both peptide-binding sites thus rendering them unusable. Once the motif-binding sites are unblocked, they can then bind to any YxxΦ or [ED]xxxL[LI] motifs in their vicinity, which, because AP2 is held against the membrane, will be those on transmembrane protein cargo. The energy liberated on cargo binding results in the further stabilization of AP2. The AP2 complex is now tightly attached to the membrane via multiple cargo and phospholipid headgroup interactions (apparent KD around 90 nM; Figure 7; Movie S3).

This model is in agreement with the stabilities of the various AP2 structures as inferred from their buried subunit interfaces (Krissinel and Henrick, 2007). In solution the locked form is more stable (10040 Å² of buried subunit interface) than the open form (9700 Å²). However, when cargo binding is taken into account, the buried interface area of the open form rises to 10590 Å² (not including bound lipids), and so the open form becomes the more stable in the presence of a PtdIns4,5P2-containing membrane. This two-state model does not exclude the existence of intermediates, such as the [ED]xxxL[LI]-liganded “unlatched” structure (Kelly et al., 2008). However, such a conformer is likely to be short-lived, as C-μ2 will be strongly attracted to the PtdIns4,5P2 of the membrane and should rapidly complete the final stages of the full conformational change to the open form, which can then bind to any available YxxΦ-containing cargo.

Recent live-cell imaging studies (Loerke et al., 2009; Saffarian et al., 2009) have revealed the presence of three types of AP2/clathrin-positive structures at the cell’s limiting membrane: those that abort in around 5 s (early abortive), those that abort within 15 s (late abortive), and those that are endocytosed at around 100 s. The model presented here is in line with these findings. The early abortive structures correspond to the situation where AP2 transiently docks to the plasma membrane but fails to undergo the activating conformational change, perhaps because the local concentration of PtdIns4,5P2 is insufficient (Figure 7, lefthand image). The endocytically productive class of structure is that in which AP2 opens and binds to cargo (Figure 7, righthand image). The late-aborted structures therefore correspond to the situation where sufficient PtdIns4,5P2 is present to drive the conformational change (Figure 7, center image) but insufficient cargo is available to further stabilize the binding of AP2 to the membrane. As we would predict from this assignment of states to conformations of our structural model, Schmid and colleagues have shown that overexpressing YxxΦ-containing cargo converts the late-abortive class of clathrin-coated structures to endocytically productive ones. This would be caused by the high concentration of YxxΦ motifs “shifting” the equilibrium between open and closed forms even further toward the open form.

Recent work has suggested that Arf6 plays a role in recruiting AP2 to the plasma membrane (Paleotti et al., 2005), despite the observation that PtdIns4,5P2 is necessary and sufficient to
efficiently recruit AP2 to the membrane. There is, however, little doubt that AP1, AP3, AP4, and COPI are all recruited to their respective membranes primarily through interactions between their large subunits and GTP-bound, membrane-associated Arf1 (Austin et al., 2000; Boehm et al., 2001; Spang et al., 1998). The high degree of structural homology between the four APs and the $\beta_1,\gamma_2,\delta_2,\zeta_2$ subcomplex of COPI (Schledzewski et al., 1999) suggests that the same gross conformational change that facilitates strong membrane attachment and cargo binding in AP2 will occur in all family members. In the case of AP2 the conformational change is driven by PtdIns4,5P$_2$ (although Arf6 may play a role), but in the case of the other AP family members the change must be driven by Arf1GTP. The most obvious way in which Arf1GTP could shift the equilibrium from the closed to the open state is by binding to and thus stabilizing only an open, cargo-binding-competent conformation very similar to that presented here for AP2. This model predicts that Arf1GTP and cargo binding would be synergistic, and this has indeed been shown to be the case for Arf1 (Bautz et al., 2006; Lee et al., 2008).

In summary, we have determined a fully ligand-bound form of AP2, which by comparison with our previous structures shows that the complex has undergone a complicated series of large-scale subunit-repositioning events. The functional need for this massive subunit rearrangement is to allow cargo binding to be coupled to PtdIns4,5P$_2$-containing membrane attachment so as to prevent inappropriate recognition of Yxx$\Phi$ and [ED]xxx[L]I sequences on cytoplasmic proteins by AP2 when it is free in the cytosol. The unblocking of both motif-binding sites is elegantly coordinated through the use of different parts of the same $\beta_2$ subunit to block simultaneously the two separate endocytic motif-binding sites.

**EXPERIMENTAL PROCEDURES**

**Structure Determination**

Recombinant AP2 cores were made as in Collins et al. (2002). Crystals of the open form were grown from a mixture of AP2 cores (7 mg/ml) with the DYQRLN peptide derived from TGN38 (2 mg/ml) by hanging drop vapor diffusion against a reservoir containing 0.7 M Lithium sulfate, 0.7 M ammonium sulfate, and 200 mM sodium citrate (pH 7.4), 5 mM DTT. Crystals were cryoprotected in mother liquor augmented with 20% glycerol and 3 mg/ml TGN38 peptide, and all data were collected on at 100K at beamline ID29 at ESRF. Crystals were of space group R3 with unit cell dimensions a = 255 $\AA$, c = 157 $\AA$. Diffraction from all crystals was severely anisotropic, extending at best to around 3.1 Å resolution in the a-b plane, but not much beyond 5 Å resolution along c. The structure was solved by multiple isomorphous replacement with anomalous scattering experimental phasing using cryo-trapped Xe (10 atmospheres of pressure for 1 min) and crystals soaked in the Ta$_6$Br$_{12}$$^{2+}$ cluster compound (Table S2). The structure was built using a combination of real-space molecular placement and reciprocal space molecular replacement guided by the experimental electron density. The structure was refined using experimental phase restraints. For a full description of the crystallographic methods and structure validation, see the Extended Experimental Procedures.

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Figure 6. Interaction of AP2 Cores with Yxx$\Phi$ and Acidic Dileucine Motifs

(A) The two motif-binding sites for Yxx$\Phi$ (gold) and acidic dileucine (yellow) are both freely accessible when AP2 is in the open conformation on the membrane. The peptides are shown attached to a modeled transmembrane helix.

(B) SPR sensorgrams of binding of AP2 core to liposomes with various compositions as indicated showing that there is no detectable binding to liposomes containing both Yxx$\Phi$ and [ED]xxx[L]I motif peptides if PtdIns4,5P$_2$ is not present.

(C) AP2 (30 $\mu$M orange) or AP2 preincubated with heparin (15 $\mu$M green) were rapidly mixed with fluorescein-labeled Yxx$\Phi$ peptide in a stopped-flow spectrometer and the change in anisotropy upon binding was measured. The anisotropy change was fitted to a single exponential function to obtain relaxation times.
Stopped-Flow Polarized Fluorescence Spectrophotometry

Binding compared to ropy). Without the addition of polymeric heparin, AP2 showed no specific of nonspecific background binding (manifested as a linear increase in anisotropy). A collimated excitation beam at 494 nm passed through a calcite prism polarizer was used to excite TgK single-mix SF-61SX2 stopped-flow spectrometer. A collimated excitation and therefore unable to bind the Yxx\textsuperscript{m} containing liposomes was recorded.

Equilibrium Fluorescence Anisotropy Measurements

The increase in fluorescence polarization anisotropy upon binding of a large molecule such as AP2 or C-\(\mu2\) to a fluorescent peptide was employed as a measure of binding. Further details are described in the Extended Experimental Procedures. Briefly, a peptide encoding the TGN38 Yxx\textsuperscript{m} motif (sequence ASDYQRL) and modified at its N terminus with fluorescein (Sigma-Genosys) was used in all equilibrium binding titration experiments at a concentration of 20 nM. Fluorescence anisotropy was measured using a PheraStar Plus plate reader (BMG Labtech) with increasing pseudo-first order concentrations of AP2, C-\(\mu2\), or \(\mu2\)-truncated core. Where used, polymeric heparin (~50 subunits) (Rovi Laboratories) was added to 500 \(\mu\)M concentration. Binding curves (Figure S7) were fitted to a single-site binding model to estimate \(K_D\). The \(\mu2\)-truncated core, lacking the C-\(\mu2\) subdomain and therefore unable to bind the Yxx\textsuperscript{m} motif, was used to determine the level of nonspecific background binding (manifested as a linear increase in anisotropy). Without the addition of polymeric heparin, AP2 showed no specific binding compared to \(\mu2\)-truncated core (Figure S7B).

Stopped-Flow Polarized Fluorescence Spectroscopy

Pre-steady-state interaction of a fluorescein-conjugated Yxx\textsuperscript{m}-containing peptide with C-\(\mu2\) and AP2 was performed using a dual-channel fluorescence TgK single-mix SF-61SX2 stopped-flow spectrometer. A collimated excitation beam at 494 nm passed through a calcite prism polarizer was used to excite 1 \(\mu\)M Yxx\textsuperscript{m}-containing peptide. Parallel and perpendicular polarized fluorescence was measured on independent photomultipliers fitted with 515 nM glass filters from which the fluorescence anisotropy was calculated. Relaxation times were determined for a range of \(\mu\)M C-\(\mu2\) and AP2 concentrations at pseudo-first order excess and used to determine kinetic rate constants (further details are given in the Extended Experimental Procedures). For heparin experiments, AP2 was preincubated for 30 min with 200 \(\mu\)M heparin.

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Accession Numbers

Coordinates have been deposited in the Protein Data Bank with PDB ID 2xa7.

Supplemental Information

Supplemental Information includes Extended Experimental Procedures, five figures, two tables, and three movies and can be found with this article online at doi:10.1016/j.cell.2010.05.006.
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