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REVIEWS

The clathrin adaptor complexes as a paradigm for membrane-associated allostery

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Abstract: The clathrin-associated adaptor protein (AP) complexes AP-1 and AP-2 are two members of a family of heterotetrameric assemblies that connect transmembrane protein cargo to vesicular coats. Cargo binding by AP-1 is activated by the small GTPase Arf1, while AP-2 is activated by the phosphoinositide PI(4,5)P_2. The structures of both AP-1 and AP-2 have been determined in their locked and unlocked conformations. The structures show how different activators use different mechanisms to trigger similar large scale conformational rearrangements. The details of these mechanisms show how membrane docking and allosteric activation of AP complexes are intimately connected.

Keywords: membrane traffic; Arf1; phosphoinositides; membrane biology; protein structure; protein crystallography

General Comments on Allostery and Membranes

This review considers a pair of multiprotein complexes whose allosteric regulation is deeply intertwined with their peripheral membrane association, the clathrin-associated adaptor protein (AP) complexes AP-1 and AP-2. Recent progress on the activation of AP-1 by the small G-protein Arf1, and AP-2 by the phosphoinositide lipid phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P_2) makes them fine examples of large scale regulatory conformational changes at membranes. The AP complexes are heterotetramers and are involved in transport processes. These are features that they share with one of the earliest paradigms of allostery, hemoglobin. Hemoglobin is an $\alpha_2\beta_2$ tetrameric complex responsible for oxygen transport.[1] A half-century ago, the Monod-Wyman-Changeux (MWC) model for allosteric binding was formulated,[2] motivated in part to account for the cooperativity of oxygen binding to hemoglobin. The analogies between hemoglobin and the AP complexes are limited to their tetrameric nature,

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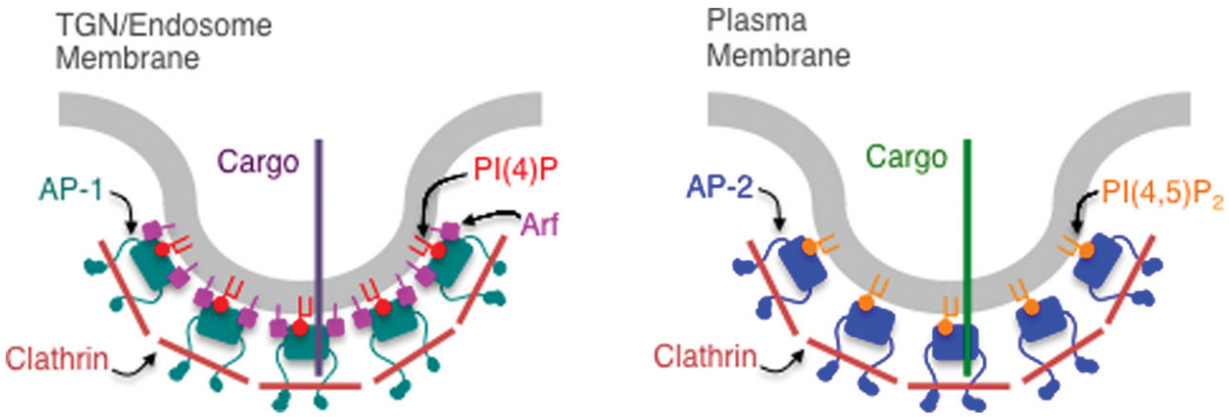


Figure 1. Clathrin coats. Schematic representation of clathrin coats containing the adaptor protein (AP) complexes AP-1 and AP-2. AP-1 is recruited to TGN or endosomal membranes by virtue of interactions with the GTP-bound form of Arf1 and PI(4)P. AP-2 is recruited to the plasma membrane mainly by interaction with $PI(4,5)P_2$. Membrane recruitment places the APs in a position to interact with sorting signals in the cytosolic domains of transmembrane cargoes and to serve as binding sites for the polymerization of clathrin into a polyhedral coat.

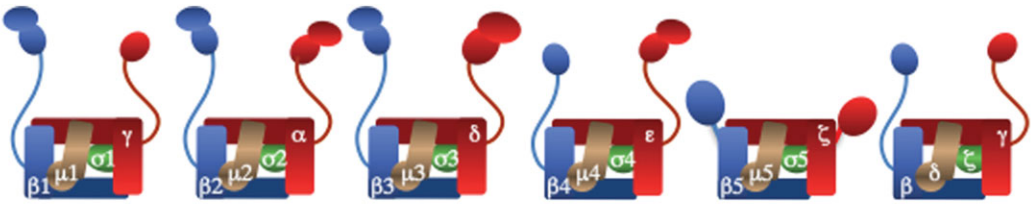
their role in transport, and their ability to adopt multiple conformations. While this review focuses on regulatory details that are unique to the AP complexes, it is amusing to see how research on two such different types of tetrameric assemblies has bracketed fifty years of progress in allostery.

Protein Coats Involved in Vesicular Transport

The interior of eukaryotic cells is organized as an array of membrane-bound compartments that is referred to as the "endomembrane system". Sequential transfer of proteins, lipids and other biomacromolecules (i.e., "cargoes") in this system occurs by budding of vesicles from a donor compartment followed by fusion of the vesicles with an acceptor compartment.[3] Transport is selective and vectorial—properties that are determined by the use of specific molecular machineries at each stage of the system. Cargo selection and vesicle budding at the donor compartment are mediated by protein "coats" that assemble at the membrane from soluble components recruited from the cytosol (Fig. 1). Several types of coat have been described, the most common of which have as its main constituent the scaffolding protein clathrin.[4] Sites of clathrin coat formation are initially marked by the presence of specific phosphoinositides and/or Arf proteins on the acceptor membrane. These molecules recruit APs, which in turn bind signals contained within the cytosolic domains of cargo transmembrane proteins while at same time serving as anchors for the polymerization of clathrin triskelia into a polyhedral coat. In essence, the APs link (i.e., "adapt") the cytosolic domains of cargoes to the clathrin scaffold, resulting in cargo capture at the forming clathrin coat. The coated membranes curve, eventually leading to the formation of a spherical clathrin-coated vesicle (CCV) that carries cargo to the corresponding acceptor compartment.

Structure and Function of AP Complexes

The first-identified and best-characterized clathrin APs are the heterotetrameric AP-1 (γ-β1-μ1-σ1) and AP-2 (α-β2-μ2-σ2) complexes (subunit composition in parenthesis). These complexes are members of a larger family of homologous complexes that also includes AP-3 (δ-β3-μ3-σ3), AP-4 (ε-β4-μ4-σ4), AP-5 (ζ-β5-μ5-σ5) and the COPI-F subcomplex (γ-COP-β-COP-δ-COP-ζ-COP)[4,5] (Fig. 2). The mammalian AP-3 complex binds clathrin,[6] although its role as a clathrin adaptor is controversial.[7] AP-4, AP-5 and COPI-F, on the other hand, are components of non-clathrin coats.[4,5] All of these complexes are structured as a large "core" domain with two long, mostly unstructured "hinge" segments, each ending in a folded "ear" or "appendage" domain (Fig. 2). Structural analyses have shown that the AP-1 and AP-2 cores consist of the N-terminal, α-helical solenoid "trunk" domains of the large subunits (γ and β1, α and β2, respectively) plus the full-length medium (μ1, μ2) and small (σ1, σ2) subunits.[4] The μ subunits comprise an N-terminal 'longin' (from 'longer N-termini') domain that is tightly bound into the core and a C-terminal domain (CTD) that swings out from the core upon activation of the complex (see below). The σ subunits consist of a single longin domain. The hinge-ear domains correspond to the C-terminal parts of the large subunits (γ and β1, α and β2).[4] The core domains mediate recruitment of AP complexes to membranes, mainly through binding to Arf proteins and/or phosphoinositides. They also harbor the binding sites for sorting signals, including tyrosine-based, YxxΦ-type signals that bind to the μ1 and μ2 CTD[8,9] and dileucine-based, (D/E)xxxL(L/I)-type signals that bind to sites at the interface of the γ-σ1 and α-σ2 subunits[10–14] (x indicates any amino acid and Φ a bulky hydrophobic amino acid). Strikingly, the core domains occur in two distinct conformations: a



Adaptor	AP-1	AP-2	AP-3	AP-4	AP-5	COPI-F
Scaffold	Clathrin	Clathrin	Clathrin?	?	SPG11?	α-β'-ε-COP
Lipid	PI(4)P	$PI(4,5)P_2$	PI(3)P	?	?	?
GTPase	Arf/Arfrp1	Arf?	Arf	Arf	?	Arf
Signals	YxxΦ [DE]xxxL[LI]	YxxΦ [DE]xxxL[LI]	YxxΦ [DE]xxxL[LI]	YxxΦ Yx[FYL][FL]E	?	?
Localization	TGN/ Endosomes	Plasma membrane	Endosomes	TGN	Late endosomes	Golgi

Figure 2. The adaptor protein (AP) complex family. Schematic representation of members of the heterotetrameric adaptor protein (AP) complex family and summary of their properties. The color scheme reflects the homology among the corresponding subunits. The listed properties include the scaffolding protein with which they associate, the GTPase and/or phosphoinositide lipid that regulate their recruitment to membranes, the sorting signals that they recognize, and their intracellular localization. SPG11 is a "clathrin-like" protein that is mutated in autosomal recessive spastic paraplegia type 11. The α-β'-ε-COP subcomplex plays a role similar to that of clathrin in the COPI coat. Sorting signal motifs are denoted according to the PROSITE pattern syntax. Φ is a bulky hydrophobic amino acid.

"locked" conformation where the binding sites for both types of signal are occluded and an "unlocked" conformation where both sites are accessible for binding.[15–18] The hinge sequences have clathrin-binding motifs that bind to the "terminal domain" of the clathrin heavy chain, while the ears bind a slew of "accessory proteins" that serve as additional adaptors, regulators of AP activation or CCV formation, or links to cytoskeletal elements.[4] The hinge-ear domains are also thought to undergo conformational changes relative to the core that regulate interactions of AP complexes with their binding partners.[19] Differential interactions with Arf and phosphoinositides, with probable contributions of accessory proteins, determine distinct intracellular localizations and functions for AP-1 and AP-2.[4] AP-1 has been ascribed multiple functions, including transport between the *trans*-Golgi network (TGN) and endosomes (in both directions) and sorting from the TGN or endosomes to different plasma membrane domains of polarized cells. AP-2, on the other hand, is exclusively involved in rapid endocytosis of a subset of transmembrane proteins from the plasma membrane.

The YxxΦ Binding Site

The structure of the YxxΦ binding site was elucidated first for the μ2 subunit of AP-2.[9] YxxΦ signals bind to the C-terminal domain of μ2, a nearly all β-sheet sandwich structure that consists of two subdomains, A and B. The YxxΦ motif binds by β-augmentation to one edge of a sheet on subdomain A [Fig. 3(A)].[9] "β-augmentation" refers to a peptide or protein binding mode involving the incorporation of the bound ligand as one or more additional strands of the β-sheet. The other key interactions are made by the Tyr residue and the hydrophobic residue Φ. The aromatic side-chain of the Tyr stacks with those of surrounding Trp, Tyr, and Arg residues, and its hydroxyl is hydrogen-bonded with a conserved Asp residue. The Φ residue is bound in a hydrophobic pocket formed at the juncture of the β-sheet sandwich. It was anticipated that the μ subunits of other AP complexes would bind YxxΦ signals in the same way, given that the interaction site is well conserved. Although there is no available crystal structure of μ1 bound to a canonical YxxΦ complex structure, a YSQA sequence from the cytosolic tail of MHC-I in complex with HIV-1 Nef binds to the equivalent site on μ1 [Fig. 3(B)].[20] Recent evidence shows that the μ3 subunit of AP-3 binds the canonical YQRL sequence from TGN at the predicted site.[21] In an exception to this pattern, an unconventional version of the signal, YKFFE, binds to the μ4 subunit of AP-4 on the opposite edge of the

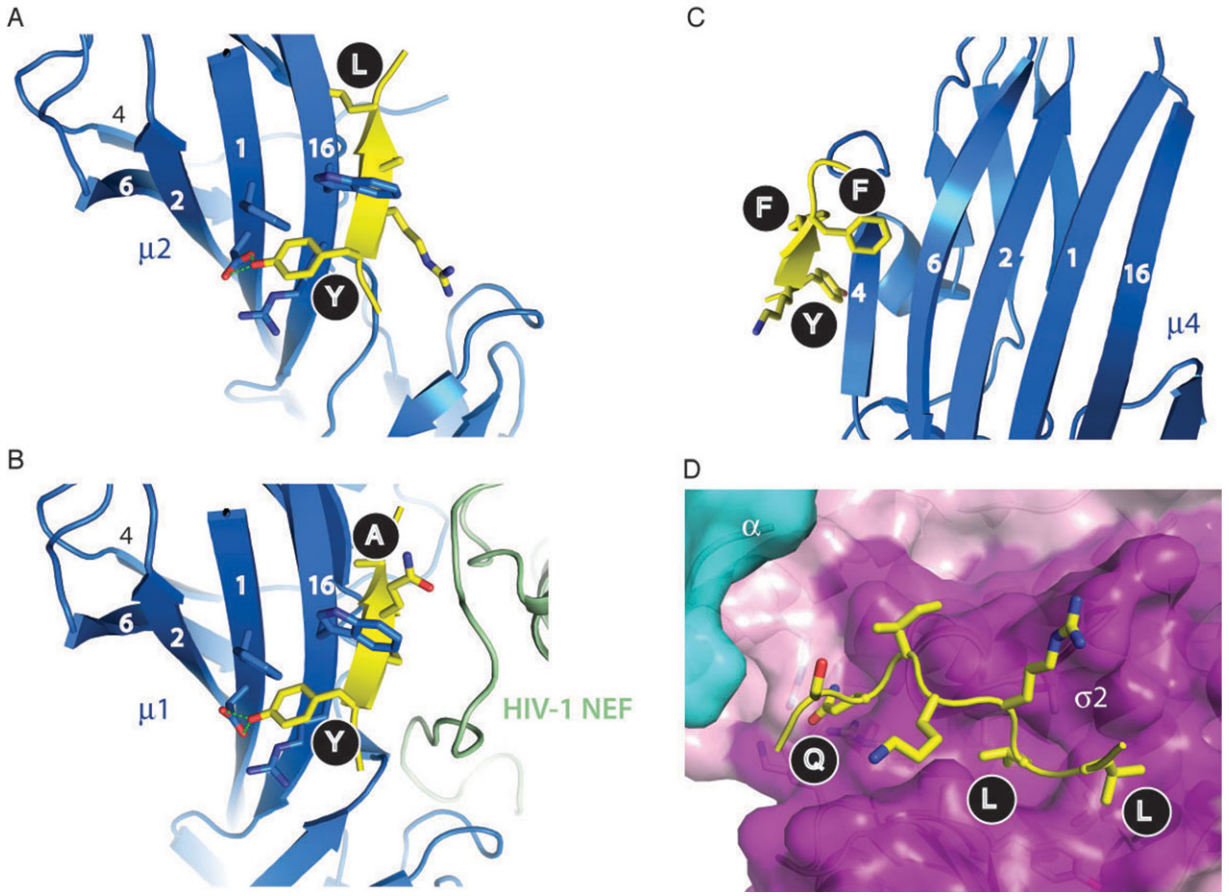


Figure 3. Cargo binding sites. A: YxxΦ signal of TGN38 bound to μ2 subunit of AP-2 by β-augmentation. μ2 in blue and YxxΦ in yellow. B: YSQA sequence from the cytosolic tail of MHC-I in complex with HIV-1 Nef bound to the equivalent site on μ1 subunit of AP-1. C: YKFFE signal binding to μ4 subunit of AP-4 on the opposite edge of the subdomain A β-sandwich. D: A variant dileucine motif from CD4 binding to the AP-2 core at the juncture between subunits σ and α.

subdomain A β-sheet sandwich [Fig. 3(C)].[22] As described below, the canonical YxxΦ signal binding site is occluded in the locked conformation of the AP complexes, while the YKFFE binding site is solvent accessible.

The (D/E)xxxL(L/I) Binding Site

The dileucine motif (D/E)xxxL(L/I) binds to the core of the AP complexes at the juncture between the σ subunit and the α, γ, or δ subunits of AP-1, -2, and -3, respectively.[13,14] The crystal structure of a variant dileucine motif from CD4, lacking the canonical Asp/Glu residue but having a phosphorylated Ser residue at position -5 from the first Leu (pSQIKRLL), was determined as bound to the AP-2 core [Fig. 3(D)].[13] The two Leu residues themselves insert their side chains into hydrophobic pockets on the surface of the σ2 subunit. The interaction with the acidic part of the signal occurs with a patch of basic residues from the N-terminal regions of σ2 and α. The AP-2 core in this structure was partially unlocked, which is required to expose the dileucine binding site. The partially unlocked structure is referred to as the "unlatched" conformation and will be described below.

Phosphoinositide Binding Sites

Phosphoinositide lipids are key markers of subcellular identity and have central roles in the organization and targeting of membrane traffic.[23] The phosphoinositide $PI(4,5)P_2$ drives the plasma membrane localization of AP-2.[24–26] To a lesser degree, another phosphoinositide, PI(4)P, is important for localization of AP-1 to the TGN/endosomes.[16,27] The most important site for AP-2 recruitment resides near the N-terminus of the α subunit [Fig. 4(A)]. The α site is bound to inositol hexakisphosphate (IP_6) in the crystal structure of the locked form of AP-2 (15). Mutational analysis shows that a closely corresponding region of the γ subunit of AP-1 binds to PI(4)P.[16] The α site and the cognate γ site are equally accessible in both the unlocked and locked conformations.

AP-2 contains a second important $PI(4,5)P_2$ binding site on subdomain B of the μ2 CTD [Fig. 4(B)]. This site is solvent exposed in both the locked and unlocked conformations. The μ2 subdomain B site does not seem to play a role in the recruitment

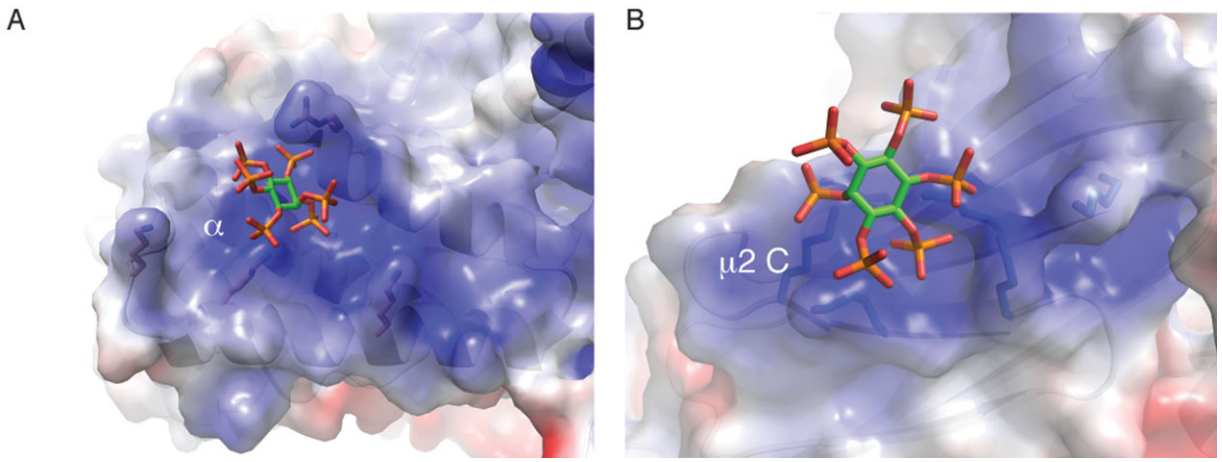


Figure 4. Phosphoinositide binding sites. A: The most important phosphoinositide PI(4,5)P_2 binding site required for AP-2 recruitment to the membrane resides near the N-terminus of α subunit. This site is bound to an inositol hexakisphosphate (IP_6) in the crystal structure of the locked form of AP-2. B: A second PI(4,5)P_2 binding site on subdomain B of the μ2 CTD. Here IP_6 molecules are shown as sticks and AP-2 as an electrostatic surface representation.

of the locked form of AP-2. This is probably because it is not coplanar with the α site, so it is not possible for the locked form to simultaneously bind two molecules of PI(4,5)P_2. Many of the basic residues in the μ2 subdomain B site are not conserved in AP-1, nor other AP complexes. Thus this site, and its role in activation, appears to be specialized to AP-2.

There is another basic patch on subdomain A of the μ2 CTD, close to the YxxΦ motif binding site.[17] This site is accessible in the locked state, despite its proximity to the occluded YxxΦ binding site. It seems to have a very minor effect on PI(4,5)P_2-dependent recruitment. It is not clear whether this is a stereospecific PI(4,5)P_2-binding site, or contributes to binding through non-specific electrostatic interactions. Finally, there is a large basic patch near the N-terminus of the β2 subunit that is occluded in the locked state by contacts with the α subunit. The β2 basic patch becomes exposed and coplanar with the other three sites in the unlocked state, and is important for recruitment to cargo-containing membranes.[17] The β basic residues are highly conserved in AP-1, although not in AP-3 or others. As with the μ2 subdomain A site, there is no direct evidence for stereospecific phosphoinositide binding by this site. Its main role could be to create an overall positive electrostatic potential in the vicinity of the membrane. The functioning of these sites in phosphoinositide activation will be described below.

μ2 Thr156 Phosphorylation

The activity of AP complexes is regulated by cycles of phosphorylation and dephosphorylation. The loop connecting the N- and C-terminal domains of μ2 is disordered in the locked conformation but folds into an α-helix in the unlocked conformation (Fig. 5).[17] This loop contains Thr156, which is a site of phosphorylation by AAK1 (α-appendage binding kinase).[28] Thr156 phosphorylation promotes cargo interactions with AP-2 *in vitro* and *in vivo*.[26,29,30] This Thr is conserved in μ1, which also undergoes phosphorylation.[31] In the unlocked state, it has been suggested that the phosphorylated Thr might make additional interactions with the β subunit, although the structure of the phosphorylated form is not known.

The Arf Binding Sites

The members of the Sar1/Arf family of small GTPases have central roles in the regulation of vesicular transport.[32,33] In particular, Arf1 is a key determinant for the identity, organization, and recruitment of effector proteins to the Golgi

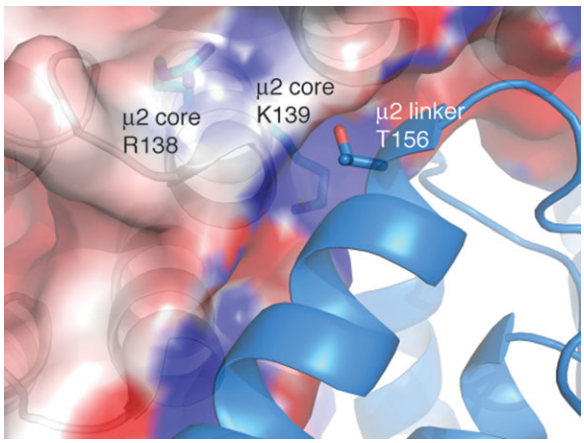


Figure 5. T156 Phosphorylation. The loop connecting the N- and C- terminus of μ2 which contains Thr156 phosphorylation site is disordered in the locked conformation folds into a helix in the unlocked conformation (shown here) of AP-2. The electropositive environment formed by μ2 core domain residues Arf138 and Lys139 is thought to favor the unlocked conformation when Thr156 is phosphorylated.

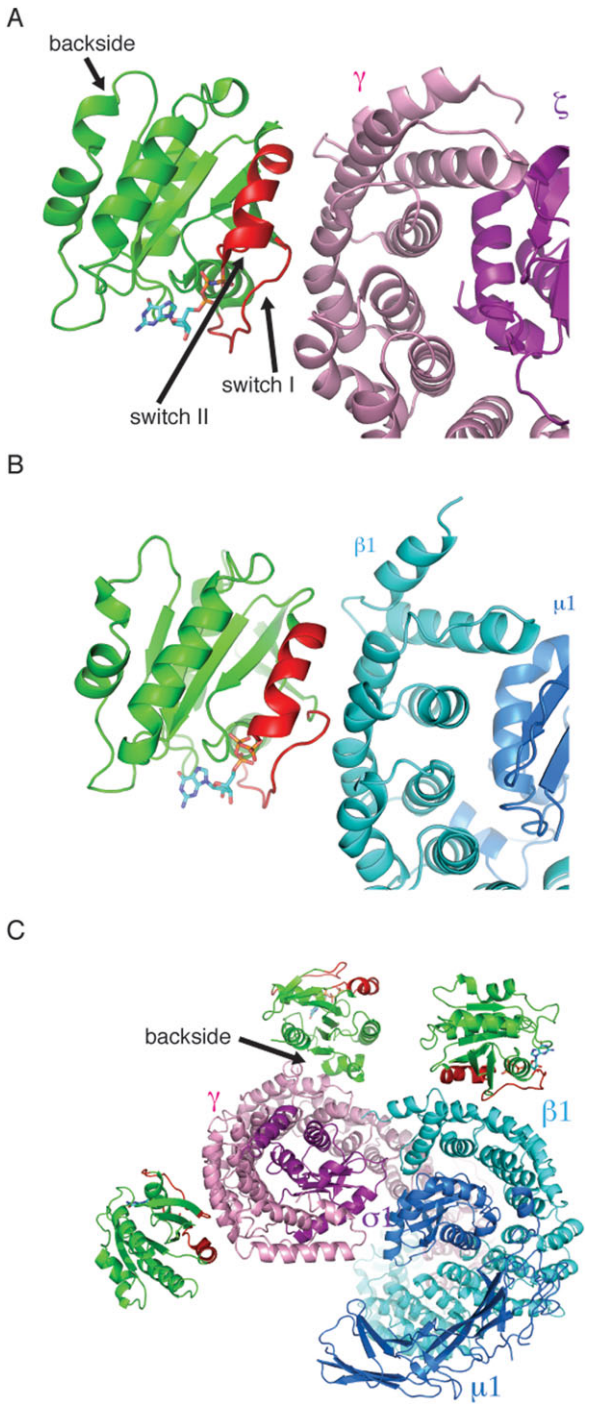


Figure 6. Arf binding sites. A: Arf1 bound to γζ-COP subcomplex of coatomer. Switch I and II of Arf1 (arrows in panel A) contact helices α4 and α6 of the trunk domain of γ-COP. The Arf1 binds to the corresponding site on the γ subunit of AP-1. B: A second Arf1 binding site with similar interactions is found on β1 subunit of AP-1. C: There are three Arf1 binding sites of AP-1. Other than the two sites shown in panels (A) and (B), there is a third Arf binding site located at the back side of γ subunit trunk, highlighted with the black arrow in (C).

apparatus. Like other GTPases, the Arf proteins cycle between a GTP-bound form that binds effectors and an inactive GDP-bound form. GTP-bound Arf proteins associate reversibly with membranes, in part due to their covalent *N*-myristoylation.

The structure of an Arf binding site on an AP complex was first described for the γζ-COP subcomplex of coatomer [Fig. 6(A)],[34] which corresponds to the γ-σ1 and α-σ2 subcomplexes of AP-1 and AP-2, respectively. Switch I and II of Arf1 contact helices α4 and α6 of the trunk domain of γ-COP. The corresponding residues on the γ subunit of AP-1 are conserved and bind Arf1.[18] The trunk domains of the large subunits of the AP complexes consist of structurally equivalent helical solenoids. These regions of the large subunits pairs (γ-β1, α-β2, γ-COP-β-COP, etc.) within the complexes have sequence identities in the <20% "twilight zone" of significance. Remarkably, a second Arf1 binding site is conserved and functional in both the β-COP subunit of COPI[34] and the β1 subunit of AP-1 [Fig. 6(B)].[18] The targeting of AP-1 to the TGN/endosomes *in vivo* requires both the β1 and γ subunit binding sites,[18] and both sites are required for maximal binding *in vitro*.[18] These two Arf1 targeting sites are conventional in the sense that the interaction occurs through switch I and II. This confers GTP dependence, but does not, by itself, account for the specificity for particular Arf proteins.

In AP-1, a third Arf1 binding site is present. This site is located on the central portion of the γ subunit trunk, and interacts with the back side of Arf1 [Fig. 6(C)]. This portion of Arf1 is distal to switch I and II. Its conformation is independent of GTP binding, while its sequence is variable in different Arf proteins. The third site does not seem to be involved in recruitment, but it is important for allosteric regulation and could contribute to Arf specificity.

The "Off State": The Locked Conformation of AP-1 and AP-2

The structure of the core of the AP class was first defined for AP-2,[15] and subsequently for AP-1.[16] In these structures, the μ CTD is lodged in a bowl formed by the trunk domains of the two large subunits. The canonical YxxΦ binding site on the μ CTD is blocked by part of the center of the β subunit trunk in this conformation [Fig. 7(A,B)]. The dileucine binding site on the α-σ2 portion of AP-2 [13] is occluded by the N-terminal part of the β trunk [Fig. 7(C,D)]. The phosphoinositide binding sites on the α subunit and the subdomain B site of μ2 C-terminal domain are exposed in this AP-2 structure, although they do not form a planar surface compatible with membrane docking [Fig. 7(E)]. Other AP-2 basic regions implicated in membrane binding are buried in this structure. All three of the Arf1 binding sites on AP-1 are solvent-exposed in the locked conformation.

Unlatching

Direct structural insight into the activation of an AP complex began with the structure of the AP-2 core

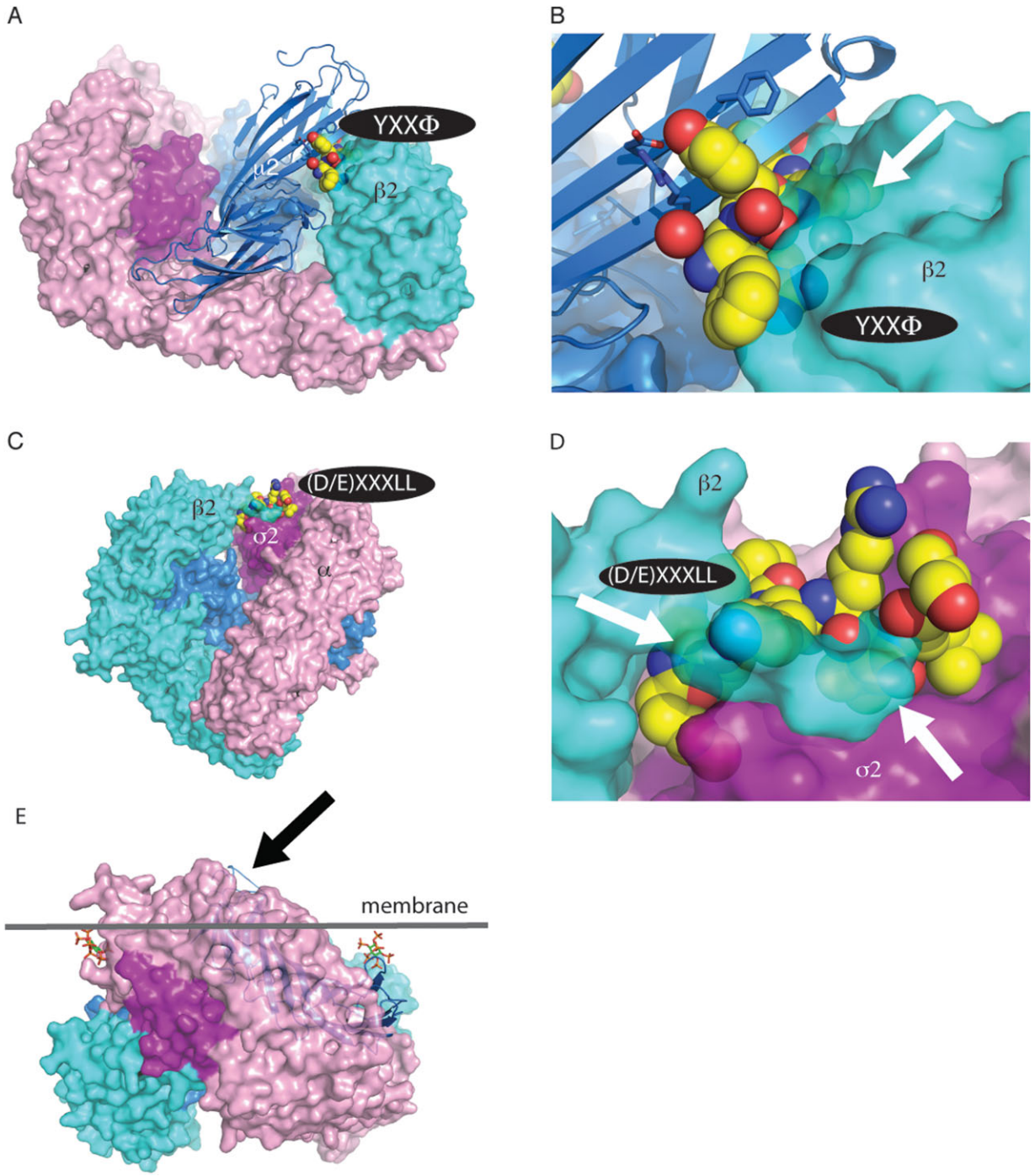


Figure 7. Cargo and phosphoinositide sites in closed state. A: The YxxΦ binding site on μ CTD is blocked by the trunk of the β subunit. The μ2 C-terminus is shown as a ribbon, and the rest of AP-2 is shown as surface, with YxxΦ shown as spheres. B: Close-up of panel (A), with the white arrow highlighting the steric overlap with the central part of the β2 subunit that prevents binding of the YxxΦ signal to the locked state. C: The dileucine biding site on σ-α2 is blocked by the N-terminus of the β subunit trunk. D: Close-up of panel (C), with white arrows highlighting steric overlaps with the N-terminal part of the β2 subunit that prevent dileucine signal binding to the closed state. E: The phosphoinositide binding sites of the locked conformation do not form a planar surface compatible with membrane docking. A large portion of the AP-2 locked conformation that sterically prevents membrane binding is shown by an arrow. The arrow highlights the collision that would occur if the locked structure were forced onto a membrane such that both available phosphoinositide binding sites were engaged.

bound to the dileucine-based endocytic signal of CD4.[13] This CD4 signal is phosphorylated at the -5 position. This CD4 peptide seems to be a potent unlatching agent in spite of the presence of a Gln instead of the normal Glu or Asp at the -4 position. The presence of an excess of this CD4 peptide in the crystallographic experiment forces binding, even though there is no $PI(4,5)P_2$ present to activate the

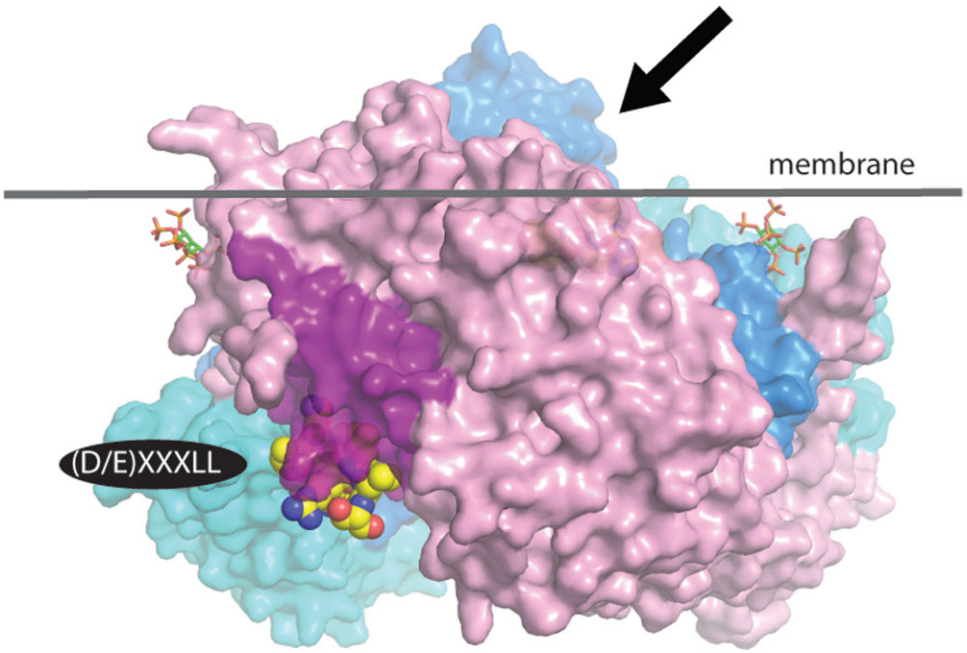


Figure 8. Partial opening of the unlatched state. A representation of unlatched state of AP-2 similar to figure 7 panel (E). The phosphoinositide binding sites are noncoplanar and not compatible with membrane docking, as also seen in the locked state. The black arrow indicates the steric collision with the membrane in this mode.

core. In turn, occupancy of this site by the peptide pries the N-terminus of the β2 subunit just far enough away from the α-σ2 subcomplex to unblock the dileucine binding site (Fig. 8). In this conformation, the canonical YxxΦ binding site is still occluded. Key lipid binding sites are either noncoplanar or completely occluded (Fig. 8). The conformation visualized in this structure is currently not thought likely to represent a physiological activated state. Rather, it seems to correspond to an intermediate state on the activation pathway.

AP-2 Unlocking by the Membrane Lipid PI(4,5)P_2

The binding of AP-2 to membrane-tethered cargo peptides is sharply increased when PI(4,5)P_2 is included in the liposomes.[17,26] This provides compelling evidence for an allosteric role for PI(4,5)P_2 in AP-2 activation. Dramatic insight into the activation pathway was obtained from the structure of the AP-2 core in an unlocked conformation induced by a high molar excess of a TGN38-derived YxxΦ motif peptide.[17] The long-chain membrane-incorporated PI(4,5)P_2 species responsible for AP-2 activation are not feasible for inclusion in conventional crystallography experiments. Indeed, the crystals used in this study do not contain any phosphoinositide compounds. Nevertheless, a PI(4,5)P_2-triggered activation mechanism was deduced from this structure and verified by *in vitro* binding experiments.[17]

The pivotal observation in the unlocked AP-2 structure is that the known PI(4,5)P_2-binding sites, other electropositive patches implicated in membrane binding, and the cargo peptide binding sites, are all coplanar with one another [Fig. 9(A,B)]. The essential role of the membrane in this activation mechanism is to present multiple molecules of PI(4,5)P_2 such that they are restricted to lie in the same plane. As the nascent CCV is formed, the plane deforms and the membrane acquires positive curvature. Given a diameter of ~70 nm, the non-planarity of the CCV is not likely to alter this principle. This model implies that soluble inositide monomers would not be capable of activating AP-2, consistent with available data.

The functions of the major PI(4,5)P_2 binding sites have been deduced from mutational analyses. Binding to the α subunit site is a prerequisite for membrane localization, making this site essential. The subdomain B site on the μ2 CTD makes little or no contribution to membrane targeting, but it is pivotally important for AP-2 activation at membranes.[17] In a simple model, the primary driving force for activation is the free energy gain from simultaneous occupancy of the α and μ2-B sites by two molecules of PI(4,5)P_2. Simultaneous binding can only occur in the unlocked state because it is only in this state that the two sites are in an unobstructed coplanar geometry. The picture is rounded out by the coplanar arrangement of two other electropositive patches, one on subdomain A of μ2, and the other on the β2 subunit.

The model that emerges from the structural and biochemical data is one of activation by coplanar presentation of multiple ligands complementary to the unlocked state. This picture is straightforward, elegant, and completely dependent on the context of

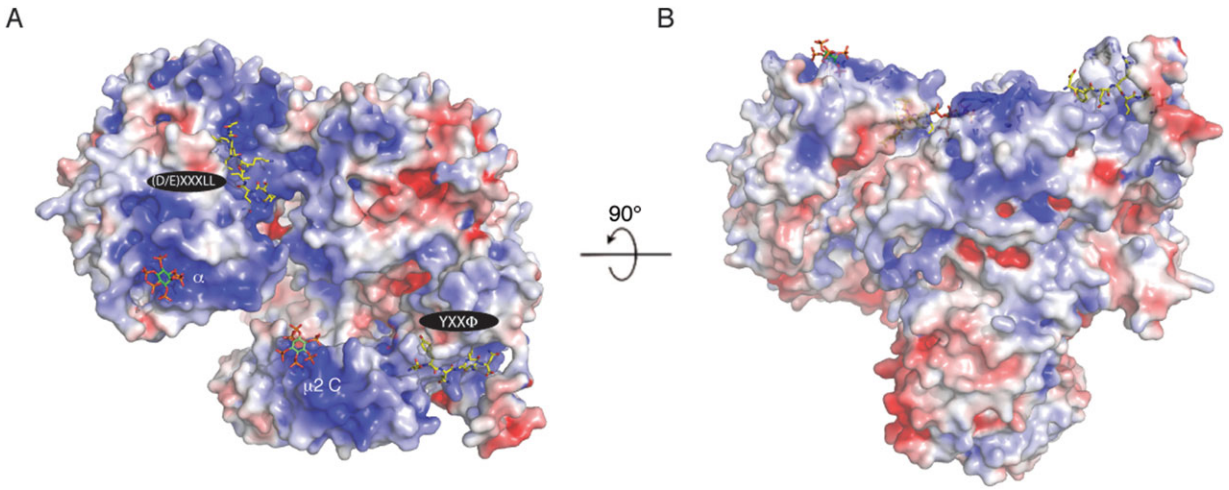


Figure 9. Unlocked AP-2. A: Electrostatic surface of AP-2 with phosphoinositides, and cargo peptides. Phosphoinositide binding sites, other electropositive patches implicated in membrane binding, and the cargo peptide binding sites are all coplanar with one another and compatible with membrane docking. B: Rotation of (A) by indicated axis.

the membrane. The model also seems to place unique constraints on the evolution of the unlocked state. To wit, the unlocked structure must be complementary in shape and electrostatics to the membrane. A multiplicity of hypothetical states could occur such that the cargo binding sites were unobstructed, but most of these would not place all of the phosphoinositide and cargo binding sites on a single face complementary to the membrane. At present, it is not clear whether more than one unlocked state actually occurs in nature.

It is interesting to consider whether the phosphoinositide activation of AP-2 has parallels in the other AP complexes. The α subunit site of AP-2 is partially conserved on the γ subunit of AP-1, where it has been implicated in binding to PI(4)P.[16] The electropositive patch on β2 is also well-conserved on β1, but other phosphoinositide and membrane binding patches of AP-2 are less conserved, if at all. As described below, most evidence suggests that the dominant pathway for AP-1 activation is via Arf1, rather than lipids. It remains to be seen to what extent membrane electrostatics and PI(4)P binding might complement Arf1 activation of AP-1 at the membrane.

The Unlocking of AP-1 by Arf1

Arf1-GTP targets most members of the AP family to intracellular membranes, including COP-I,[35] AP-1,[36,37] AP-3,[6] and AP-4.[38] Arf1 does more than just target AP complexes, however. The binding of cargo peptides and Arf1-GTP is highly synergistic, suggesting that Arf1 induces an allosteric change in AP-1.[18,39] Crystallographic analysis of an AP-1:Arf1 complex showed that the AP-1 core was in an unlocked conformation identical to that of the unlocked form of AP-2.[18] This conformational change does not require lipids or peptide signals. Thus, the mechanism of coplanar membrane presentation and recognition that was described above for AP-2 cannot explain the activation of AP-1 by Arf1. None of the Arf1 binding sites on the AP-1 core are occluded in the locked state, which rules out intrasteric competition as an activation mechanism. Arf1 binding does not seem to induce any dramatic localized conformational changes in the subunits to which it binds, although this latter conclusion is tempered by the low resolution of the AP-1 analysis. These observations seem to rule out most of the obvious precedents, and suggest that something out of the ordinary is at work.

The crystallized form of the unlocked AP-1:Arf1 complex consists of a dimer in which two copies of the unlocked state of AP-1 are bridged by two molecules of Arf1 (top and botton in Fig. 10). To gain insight into the transition path between the locked and unlocked states, a hypothetical model was constructed by placing two copies of the locked form of AP-1[16] into the lattice of the Arf1-bound crystallographic dimer [Fig. 10(A)].[18] In this locked model, it is not possible to make a complete set of contacts with Arf1, so this model lacks the contact between the left-hand AP-1 and the bottom Arf1. Formation of a full set of Arf1 contacts as seen in the crystallographic unlocked AP-1 dimer triggers a chain of events leading to the unlocking of the complex [Fig. 10(A–E)]. To engage switch I and II of the bottom Arf1 molecule, the β1 trunk pivots toward it [Fig. 10(B)]. This pivot swings the C-terminal part of the β1 trunk towards the μ1 CTD such that part of μ1-CTD collides with the β1 trunk [black arrows, Fig. 10(F,G)]. This ejects μ1-CTD from the bowl [Fig. 10(C)]. At the same time, the γ subunit of the right-hand AP-1 pivots towards the β1 subunit of the left-hand AP-1 finish forming the Arf1 contact [Fig. 10(C)]. This pivot motion squeezes the μ1 CTD from

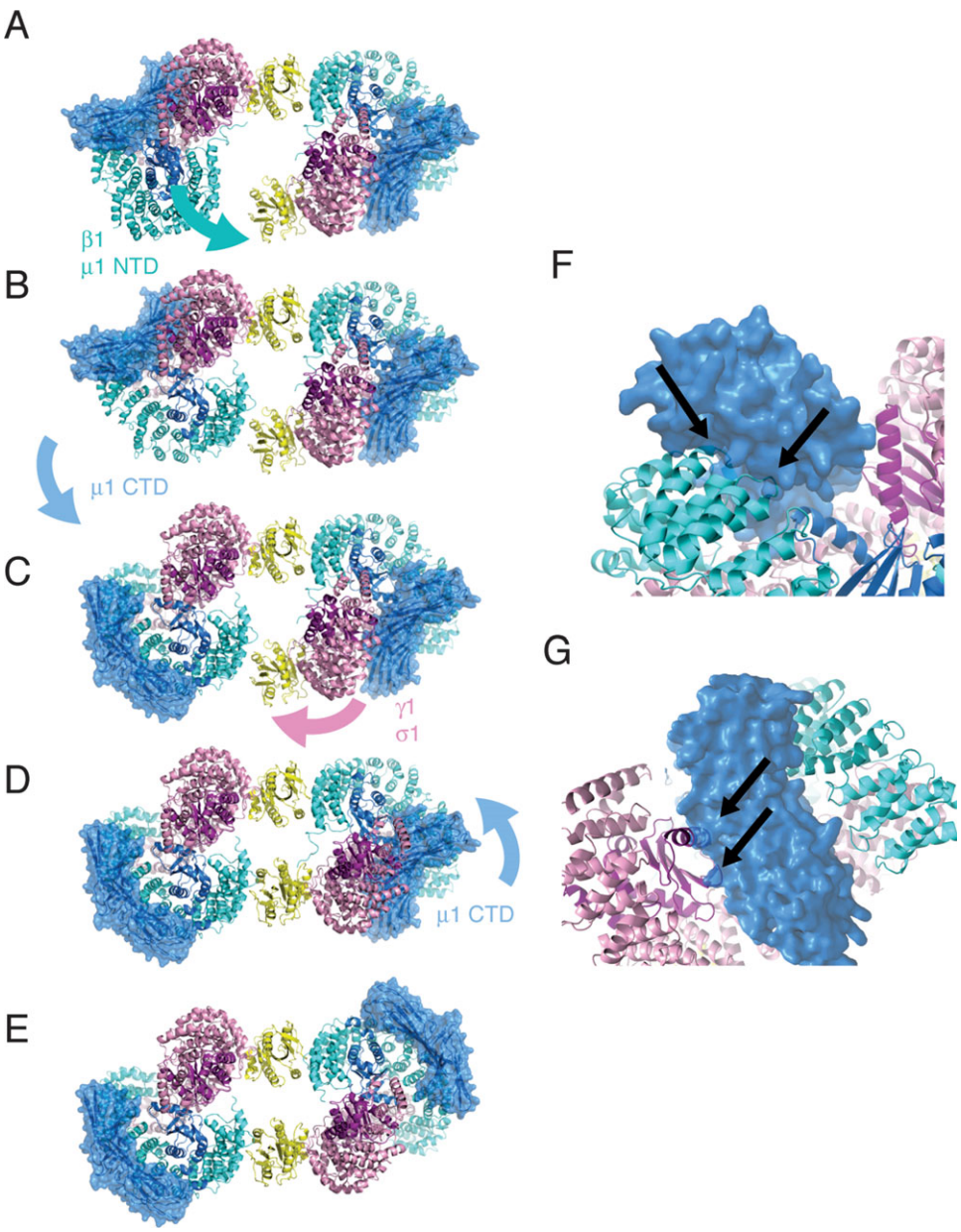


Figure 10. How Arf1 unlocks AP-**1**. Animation of a speculative model for the step-by-step activation of AP-1 by Arf1 binding. Hypothetical intermediate states have been interpolated between the observed locked and unlocked structures to illustrate one potential unlocking pathway. Colored arrows indicate movements of the associated subcomplexes or domains. Black arrows in (F) and (G) denote steric collisions introduced by pivoting of the trunks. CPK models are shown for the dileucine signal binding to the core followed by YxxΦ signal binding to the μ1 CTD. An interactive view is available in the electronic version of the article.

the γσ1 side of the bowl [Fig. 10(D)], ejecting the right-hand μ1-CTD into the unlocked conformation [Fig. 10(E)].

The net result of the Arf1-triggered pivoting is to bring the central portions of the β1 and γ trunks closer to each other. This constricts the bowl between the β and γ subunits, narrowing it from 40 Å to 30 Å. The compression of the bowl destroys the binding site for the μ1-CTD and forces it into the unlocked state. An analogy can be made to a pair of scissors, where the 20 Å unlocking of the handles by the two copies of Arf1 leads to the closing of the blades by 10 Å about the position of the μ1A CTD, forcing it out of the locked conformation. While the details described above for the transition pathway for Arf1-induced activation are speculative, the essence of the activation mechanism is still relatively straightforward. The bridging of the AP-1 dimer by the two molecules of Arf1 is incompatible with the locked state, while it stabilizes the unlocked state through the binding energy contributed by forming an additional AP-1:Arf1 contact.

The key evidence for the Arf1-induced dimerization model for AP-1 activation comes from

mutational disruption of the backside of Arf1. This contact forms half of the dimeric bridge, together with the canonical switch I and II interface. Mutation of the backside greatly diminishes the ability to Arf1 to promote dileucine cargo binding to AP-1.[18] On the other hand, the same backside mutation actually increases the affinity of Arf1 for AP-1. While this increase in affinity might seem counterintuitive, it is consistent with the coupling of Arf1 backside binding to an energetically costly conformational change. In contrast to the activation of AP-2 by $PI(4,5)P_2$, which is inherently dependent on the membrane-bound presentation of the activator, Arf1 can activate AP-1 *in vitro* without membranes. The simultaneous docking of the AP-1:Arf1 dimer to membranes and cargoes is compelling, however. The cooperative engagement of cargo, membrane, and Arf proteins is almost certainly essential for AP-1 activation *in vivo*. For practical purposes then, the activation of AP-1 can be considered almost as deeply intertwined with the membrane as for AP-2.

Future Perspectives

It will be important to determine to what extent the Arf- and phosphoinositide-dependent activation mechanisms apply across the AP family. Many of the interaction sites involve multiple residues that are conserved to varying degrees, which complicates attempts to predict their properties from conservation. The low resolution of the available AP-1:Arf1 complex adds to the ambiguity. For AP-1 and Arf1, the details of the side-chain interactions between them have been inferred from the overall positioning of the subunits but not visualized directly. While PI(4)P plays a role in recruiting AP-1 to the TGN, it has not been possible to demonstrate an allosteric role for the phosphoinositides in AP-1 activation.[18] The $PI(4,5)P_2$ binding residues of AP-2 are only partially conserved in AP-1, and less conserved in other members of the family, suggesting this mode of activation is probably specialized to AP-2.

Targeting by bivalent binding to Arf1 via the β and γ subunits is a confirmed property of both AP-1 and COP-I. What is not clear is whether any of the AP family members apart from AP-1 are subject to allosteric activation. Higher resolution analysis of the AP-1:Arf1 complex will be helpful, while direct biochemical investigation will be essential to clarify these points. The situation with AP-2 is intriguing. AP-2 is not known to be regulated by Arf1, but the plasma membrane associated Arf6 has been suggested to regulate AP-2.[40–43] The switch I and II regions of the Arf proteins are highly conserved with one another, and the Arf1 binding sites on AP-1 β1 and γ subunits are partially conserved on the AP-2 β2 and α subunits. The possibility of allosteric regulation of AP-2 by Arf proteins has not received much attention, but this will be a significant point to clarify going forward.

The conformational changes described in this review concern the AP core. The core assembly has been the focal point here thanks to the advances in crystallization of the AP-1 and AP-2 cores. The larger context of the full AP complex, including the linkers and appendage domains of the two large subunits, will be very important to understand. Binding of the CI-MPR dileucine signal to AP-1 promotes tryptic cleavage of the hinge between the trunk and appendage domains of AP-1 β1, for example.[39] This indicates that the conformation of the core controls the conformation of the hinge, and in turn, the position of the appendage domain. The hinges contain the clathrin binding motif that binds to the terminal domain of the clathrin heavy chain.[44] Clathrin binding to the hinge, outside of the core domain, helps unlock the AP-2 core as judged by its ability to be recruited to the $PI(4,5)P_2$-containing plasma membrane.[45]

Moving up in size and complexity, the full-length AP complexes function in the context of the nascent coat, consisting of AP complexes and clathrin, or coatomer. The coat assembles concomitantly with the induction of membrane curvature as the vesicle is formed. There is currently no reason to think that the locked or unlocked conformations of AP core have any ability to sense or promote curvature on their own. However, Arf proteins have been implicated in regulating membrane curvature[46,47] and it is worth considering how coassembly with AP complexes might affect this. In the presence of membranes, the N-terminal region of Arf1-GTP folds into a helix[48] and is believed to wedge itself into the membrane interface, promoting positive curvature.[46,47] As a consequence of binding at least two copies of Arf1, the two N-terminal membrane-bound helices are brought within ~70 Å of one another. This is closer together than the ~100 Å between the H0 helices of N-BAR domains, which are highly effective at promoting curvature.[49] The potential role of AP-1-dependent Arf1 clustering in promoting membrane curvature will be important to explore, as it might explain why the dual Arf1 targeting mechanism appears to be conserved among at least a subset of AP complexes.

There is no reason to assume that the list of allosteric modulators of AP conformation is restricted to phosphoinositides, Arf proteins, cargo, phosphorylation, and clathrin. The Nef protein of HIV-1 has been implicated in stabilizing the association of AP-1 with membranes,[50] for example. There is also no reason to expect that the known locked, unlatched, and unlocked states define the entire conformational space accessed by the AP complexes. The Arf family protein Arfrp1 appears to have a unique ability to selectively promote the binding of AP-1 to Vangl2, a

signaling factor of polarized epithelial cells that contains a YYXXF sorting signal.[51] Arf1 lacks this activity, despite its ability to promote exposure of the canonical YxxΦ binding site. One appealing model is that the YYXXF binding site, which might be distinct from the canonical YxxΦ binding site, becomes exposed in a novel conformation promoted by Arfrp1 but not Arf1. It is striking that thus far, the locked and unlocked conformations of AP-1 and AP-2 correspond so closely to one another. This should not lead us to neglect that a spectrum of other physiological conformations may exist that have yet to be visualized.

This review began by drawing a comparison between the allosteric regulation of heterotetrameric AP cores and hemoglobin. These systems differ in many ways and resemble one another in just a few. Nevertheless, experiences with hemoglobin, one of the first model systems for understanding allostery, are instructive for the next steps in mechanistic analysis of AP complex regulation. Given that the AP-1 and AP-2 cores have been made tractable to x-ray crystallography, single molecule spectroscopic studies are clearly feasible. This type of experiment should lead to important insights into the conformational dynamics and transitions. Molecular simulations will also be invaluable for understanding the detailed mechanics of the unlocking transition. In this arena, increases in computational capacity, together with the crystallographic definition of locked and unlocked states for both AP-1 and AP-2, promise much in the way of progress and insights to come.

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