Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits.

I Gaidarov  
*Thomas Jefferson University*

James H. Keen  
*Thomas Jefferson University, James.Keen@jefferson.edu*

Let us know how access to this document benefits you

Follow this and additional works at: [https://jdc.jefferson.edu/mifp](https://jdc.jefferson.edu/mifp)  

Part of the [Medical Microbiology Commons](https://jdc.jefferson.edu/mifp)

Recommended Citation  
[https://jdc.jefferson.edu/mifp/46](https://jdc.jefferson.edu/mifp/46)
Phosphoinositide-AP-2 Interactions Required for Targeting to Plasma Membrane Clathrin-coated Pits

Ibragim Gaidarov and James H. Keen
Kimmel Cancer Institute and the Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Abstract. The clathrin-associated AP-2 adaptor protein is a major polyphosphoinositide-binding protein in mammalian cells. A high affinity binding site has previously been localized to the NH₂-terminal region of the AP-2 α subunit (Gaidarov et al. 1996. J. Biol. Chem. 271:20922–20929). Here we used deletion and site-directed mutagenesis to determine that α residues 21–80 comprise a discrete folding and inositide-binding domain. Further, positively charged residues located within this region are involved in binding, with a lysine triad at positions 55–57 particularly critical. Mutant peptides and protein in which these residues were changed to glutamine retained wild-type structural and functional characteristics by several criteria including circular dichroism spectra, resistance to limited proteolysis, and clathrin binding activity. When expressed in intact cells, mutated α subunit showed defective localization to clathrin-coated pits; at high expression levels, the appearance of endogenous AP-2 in coated pits was also blocked consistent with a dominant-negative phenotype. These results, together with recent work indicating that phosphoinositides are also critical to ligand-dependent recruitment of arrestin-receptor complexes to coated pits (Gaidarov et al. 1999. EMBO (Eur. Mol. Biol. Organ.) J. 18:871–881), suggest that phosphoinositides play a critical and general role in adaptor incorporation into plasma membrane clathrin-coated pits.

Key words: clathrin • adaptor • phosphatidylinositols • endocytosis • adaptins

RECEPTOR-MEDIATED endocytosis is a multistep process by which certain cell surface proteins are specifically and efficiently internalized into cells through plasma membrane coated pits. Clathrin, the major structural component of the cell surface coated pit, is a triskelion-shaped protein that forms the regular polygonal surface lattice of the coat and provides its structural integrity. A nonglobular protein component of the coat is the multimeric protein complex termed adaptor or A P1, for assembly or associated proteins. Most of the APs are heterotetrameric proteins and multiple forms have been identified (reviewed in Schmid, 1997). The best characterized of these are the AP-1 and AP-2 proteins, which are involved in clathrin coat formation and sorting at the Golgi and plasma membrane, respectively. These AP molecules consist of two large subunits (γ and β' in AP-1 and α and β in AP-2), a medium subunit (μ1 in AP-1 and μ2 in AP-2), and a small subunit (σ1 in AP-1 and σ2 in AP-2). The α subunits have some homology to the γ subunit and both are very distantly related to the β/β' subunits (reviewed in Keen, 1990; Schmid, 1997).

AP-2 is critical for two of the key functions of the early steps of the endocytosis pathway: the formation of the clathrin lattice and selection of specific cargo proteins for internalization. A P-2 interacts with clathrin through the α and β subunits (Goodman and Keen, 1995; Shih et al., 1995) and promotes coat formation (Prasad and Keen, 1991). Interaction of the AP-2 μ subunit with receptors containing tyrosine-based internalization motifs contributes to their localization to coated pits (Ohno et al., 1995; Sorkin et al., 1995; Boll et al., 1996; Heilker et al., 1996; Shiratori et al., 1997), and there is evidence that AP-2 may interact with other classes of internalization signals as well (Rapoport, 1998; Rodionov and Bakke, 1998).

The function of AP-2 in endocytosis is probably modulated by multiple factors. Protein–protein interactions of AP-2 with other macromolecules implicated in the endocytosis pathway such as dynamin (Wang et al., 1995), synaptotagmin (Zhang et al., 1993), amphiphysin (M. et al., 1996), Eps15 (B. et al., 1996; Iannolo et al.,
Materials and Methods

Materials

Clathrin was purified from bovine brain–coated vesicles as described (Kee, 1987). o-myo-Inositol hexakiphosphate (IP₆) was obtained from Calbiochem. [³H]IP₆ was from DuPont NEN. [³⁵S]-Translabel was from ICN. l-3-3-tosylamido-2-phenylamino chloromethyl ketone-trypsin was from Worthington Biochemical, Inc. Restriction and modification enyzmes were purchased from Boehringer Mannheim. S-Sepharose and Sepharose 2B were from Sigma Chemical Co. Tnt rabbit reticulocyte transcription-translation system was from Promega. All other chemicals were reagent grade or better and were purchased from Sigma Chemical Co. or Fisher.

Construction of Deletion and Site Mutants in Maltose-binding Protein αα5-80

Deletion and site-directed mutations in the αα5-80 insert in the plasmid pH M.5-80 (Gaidarov et al., 1996) were performed using a combination of subcloning procedures and polymerase chain reaction. Details of these procedures are available upon request. The fusion proteins were expressed and purified as described previously (Gaidarov et al., 1996).

Preparation of Wild-type and Mutant αα5-80 Peptides

Purified maltose-binding protein chimerae were dialyzed into 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM CaCl₂, and digested with the Factor Xa (1 mg enzyme/200 mg fusion protein) for 24–36 h at 4°C. The digestion mixture was then incubated with amylase beads to remove MBP and undigested fusion protein. The supernatant was then applied to an S-Sepharose column (Pharmacia) and the column was washed with 20 mM sodium phosphate, pH 7.3, 200 mM NaF. The bound peptide was eluted with 20 mM sodium phosphate and 1 M NaF. The eluate was dialyzed into 10 mM sodium phosphate, pH 7.3, and 100 mM NaF, and used for circular dichroism spectroscopy. Purity of the peptide was checked by SDS-electrophoresis on an 8–25% gradient gel using a PhastSystem (Pharmacia).

Inositol Polyphosphate-binding Assay

The binding of [³H]IP₆ to recombinant fusion proteins was determined by a polyethylene glycol precipitation procedure as described previously (Gaidarov et al., 1996).

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was performed on an Aviv-620S instrument at ambient temperature under nitrogen atmosphere with peptides at 0.1–0.4 mg/ml in 10 mM sodium phosphate, 100 mM NaF. The CD spectra were analyzed using secondary structure prediction software based on the method described by A ngrade et al. (1993).
pBluescript SK – (R Robinson, 1993) was digested with EcoRI, treated with Klenow fragment and dNTP, and subsequently digested with SacI. Excised fragments were ligated into the plasmid pSP65 that had been digested with SacI and Smal, resulting in the plasmid pSP65-dN-αγa. This plasmid was cut with SacI and Smal, and the excised fragment was ligated together with the linker connecting Ndel and SacI sites into the plasmids pSP65α2 and pSP65 α5 KKQ from which Ndel-Smal fragments were excised. The resultant plasmids pSP65-Wγγa and pSP65-M18γγa contained wild-type and mutant αγa chimeric inserts. These plasmids were consecutively treated with Smal, bluntend with Klenow fragment and dNTP, and then digested with EcoRI. The excised inserts were ligated into eu- karyotic expression vector pCDNA 3 that had been cut with EcoRI and EcoRV, resulting in plasmid constructs pCDNA 3α5γγaWT and pCDNA3- α5γγaKKQ.

For transient expression, BALB/c 3T3 cells were grown in T-75 flasks in a humidified atmosphere with 5% CO2 supplemented with 5% fetal bovine serum, 5% calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were grown to 60-70% confluence and transfected with expression constructs using Lipofectamine reagent. In brief, 15 μg of pCDNA 3α5γγaWT or pCDNA 3α5γγaKKQ were incubated with 80 μl of Lipofectamine in 5 ml of DMEM for 30 min at room temperature. A flter the incubation 5 ml of DMEM was added and the mixture was transferred to a 24-well plate. Immunofluorescence analysis was performed 48 h after transfection.

Results

Dissection of the AP-2 α PPI Binding Site by Truncation Mutagenesis

In our previous study, using photoaffinity labeling and bacterially expressed fusion proteins, we localized the high affinity PPI binding site on the clathrin adaptor protein AP-2 to the region between residues 5 and 80 at the NH2-terminal of the α subunit. To determine whether PPI binding could be localized to a shorter sequence within this region, we produced several maltose-binding protein (MBP) fusion proteins containing smaller fragments (Fig. 1). A mong fusion proteins containing either residues 5-21, 21-80, 5-49, or 50-80, only those containing residues 21-80 retained specific IP6 binding, with affinity similar to that of the full fragment 5-80 (Fig. 1); the other fusion proteins did not display any detectable binding. This suggested that the PPI binding site in the A P-2 α subunit may not be represented by a short stretch of residues, but that a relatively large portion of the sequence between amino acids 21-80 may be required to form a discrete domain with proper tertiary structure.

Site-directed Mutagenesis of the AP-2 α PPI Binding Domain

The A P-2 α sequence between residues 5 and 80 is a fairly basic region with several clusters of cationic residues. It is likely that positive charges are involved in the interaction with the negatively charged phosphate groups of PPIs. We investigated more closely the role of these basic residues in IP6 binding. A ccordingly, we produced a series of fusion proteins of MBP with the A P-2 α5-80 fragment (de- noted M BP-α5-80) in which each basic amino acid (10 lysines and 4 arginines) was changed to a glutaminyl residue. Glutamine was chosen because it contains a substantial side chain, similar to lysyl and arginyl residues, but is uncharged. Each of these fusion proteins was purified by affinity chromatography and tested for IP6 binding.

We found that residues scattered throughout the α 5-80 region affected IP6 binding, though to differing extents (Fig. 2). The mutations could be divided into several groups in terms of their effects on IP6 binding: no reduc-
tion in IP₆ binding activity (R21); slight (~20%) reduction (K24, K26, R41, K43, K48); substantial (~40%) reduction (K31, R32, K35, K45, K61); and large (>60%) inhibition (K55, K56, K57). To investigate further the role of lysyl residues 55–57 whose alteration to glutamines had the most pronounced effect on IP₆ binding, we generated an additional mutant in which residue K56 was changed to glutamic acid with reversal of charge. The IP₆ binding ability of this mutant was even more greatly diminished, to ~30% of the wild-type protein, compared with substitution with glutamine. When all three lysyl residues were changed to glutamines in a single mutant, denoted KKK/Q, the IP₆ binding ability was decreased to <10% of the wild-type protein. This mutant, essentially devoid of IP₆ binding, was characterized further using biophysical methods and functional assays described below to determine whether these residues are directly involved in PPI binding, or whether the decrease in PPI binding is the result of gross conformational change in the structure of the protein.

Structural Analysis of the Wild-type and Mutant AP-2 α PPI Binding Domains by Circular Dichroism Spectroscopy

CD spectroscopy is a very useful method for the rapid determination of secondary structures of peptides and proteins. We employed CD spectroscopy to characterize the secondary structure of the A P-2 PPI binding domain and to monitor any changes resulting from the mutations introduced in its sequence. The A P-2 α5-80 fragments of the wild-type and KKK/Q mutant fusion proteins were cleaved with Factor Xa and purified from MBP by consecutive affinity and ion-exchange chromatography steps as described in Materials and Methods. The purification procedure resulted in peptide preparations that were uniformly ~95% homogeneous (data not shown). A CD spectrum of the isolated wild-type A P-2 α5-80 fragment is shown in Fig. 3a. The positive absorption peak at 195 nm and two negative peaks at 207 and 222 nm indicate that the conformation of the α5-80 peptide has substantial α-helical content. Secondary structure calculated by the method of Andrade et al. (1993) yielded estimates of ~37% α-helix and 26% β sheet. A iso shown in Fig. 3a is the CD spectra of the wild-type A P-2 α5-80 fragment in 50% trifluoroethanol, known to induce an α-helical conformation in oligopeptides (Sonichsen et al., 1992). Under these conditions, the spectrum of the wild-type fragment exhibited much more pronounced minimum and minima, corresponding to almost 100% α-helicity.

The CD spectrum of the mutant A P-2 α5-80-K K K/Q fragment is presented in Fig. 3b, along with the spectrum of the wild-type peptide. The mutant α5-80-K K K/Q has secondary structure content practically identical to that of the wild-type protein. The CD spectrum of the purified peptide derived from the charge inversion mutant A P-2 α5-80-K 56E was also indistinguishable from that of the wild-type (data not shown). These results argue that the decrease in PPI binding observed with these mutants is not the result of gross conformational changes induced by the
corresponding to the NH$_2$-terminal core and COOH-terminal appendage domains, virtually identical to that of the wild-type polypeptide (Fig. 4a). This result demonstrates that alteration of the K(55-57) residues in the NH$_2$-terminal region of the AP-2 polypeptide does not cause gross misfolding of the entire subunit on synthesis.

The isolated A-P-2 $\alpha$ subunit generated by in vitro translation has also been shown to bind specifically to clathrin (Goodman and Keen, 1995). This provided a useful assay to ask whether lysyl residues 55-57 were directly involved in clathrin binding, or whether their substitution with glutamines altered structural properties of the isolated A-P-2 $\alpha$ polypeptide required for this interaction. We found that binding of the in vitro-translated mutant KKK/Q $\alpha_A$ polypeptide produced a pattern with the characteristic core and appendage domains, virtually identical to that of the wild-type polypeptide (Fig. 4a). This result indicates that alteration of the K(55-57) residues in the NH$_2$-terminal region of the A-P-2 $\alpha$ polypeptide does not cause gross misfolding of the entire subunit on synthesis.

**Figure 4.** A-P-2 $\alpha$ and the KKK/Q mutant lacking PPI binding have similar domain structure and clathrin binding activity. (a) In vitro translated wild-type A-P-2 $\alpha$ subjected to limited proteolysis with 0, 50, 100, or 150 ng/ml trypsin (lanes 1-4, respectively) for 15 min at room temperature results in generation of fragments corresponding to the 60-66-kD core (C) and 40-kD appendage (A) domains, following preferential cleavage in the hinge region as previously described (Goodman and Keen, 1995). The cleavage pattern of the mutant A-P-2 $\alpha$ KKK/Q translation product is virtually identical in terms of both protease sensitivity and fragments generated, indicating the presence of similar folded domains. (b) Most of input (I) of in vitro-translated wild-type A-P-2 $\alpha$ binds to clathrin cages (30 $\mu$g) and becomes sedimentable in their presence (+), while little sediments in their absence (−), as previously reported (Goodman and Keen, 1995). The mutant A-P-2 $\alpha$ KKK/Q translation product shows essentially identical behavior.

Functional Characterization of Mutant KKK/Q AP-2 $\alpha$ in Intact Cells

To investigate the functional role of the A-P-2 PPI binding site in intact cells, we employed an $\alpha_{\gamma C}$ construct described previously (Robinson, 1993) and kindly provided by Dr. M.S. Robinson (University of Cambridge). The $\alpha_{\gamma C}$ construct encodes mouse $\alpha_C$ polypeptide in which the hinge region between the core and appendage domains, corresponding to $\alpha_C$ residues 620-700, has been substituted with the hinge region of the bovine Golgi-specific A-P-1 $\gamma$ subunit. This enabled us to specifically localize the expressed $\alpha_{\gamma C}$ polypeptides in transiently transfected mouse fibroblasts using a $\gamma$-specific monoclonal antibody (mAb 100/3) which does not recognize the endogenous (mouse) protein (Ahele et al., 1988). Additionally, the endogenous A-P-2 $\alpha$ polypeptide could also be uniquely localized using A.b31, a rabbit polyclonal anti-$\alpha$ antibody kindly provided by Dr. A. Sorkin (University of Colorado). We found that though A.b31 was produced by inoculation with a fragment consisting of the hinge and appendage domains of the rat brain $\alpha_C$ subunit (Sorkin et al., 1995), it reacts only with the $\alpha$ hinge region and not with the $\alpha$ appendage (see Materials and Methods). Thus, the endogenous and the transiently expressed exogenous $\alpha$ polypeptides could be detected independently, providing important tools for the study of mutant $\alpha$ subunits.

First, we asked whether wild-type and KKK/Q mutant A-P-2 $\alpha_{\gamma C}$ polypeptides expressed after transfection are incorporated into A-P-2 adaptor complexes in intact cells. Lysates of mock, wild-type $\alpha_{\gamma C}$, and KKK/Q mutant $\alpha_{\gamma C}$-transfected MOP8 mouse fibroblasts were challenged with monoclonal antibody 100/3, and the resultant immunoprecipitates were fractionated by SDS-PAGE and analyzed by immunoblotting with antibodies to the other A-P-2 subunits. As shown in Fig. 5, reactivity with the anti-$\gamma$ 100/3 antibody was detected only in immunoprecipitates from cells transfected with the wild-type or mutant $\alpha_{\gamma C}$ constructs, consistent with the inability of this antibody to recognize the endogenous mouse A-P-2 $\gamma$ polypeptide. On im-
munoblotting with antibodies to the β, μ2, or α2 subunits of AP-2, no signal was detected in the immunoprecipitates from mock-transfected cell lysates demonstrating that recovery of the endogenous AP-2 subunits were dependent on their incorporation into complexes containing exogenous αγα polypeptide. However, anti-γ immunoprecipitates of cells transfected with either the wild-type or the KKK/Q αγα constructs contained the endogenous β2, μ2, and α2 subunits in similar amounts. These findings confirm the results of Page and Robinson (1995) in indicating that the wild-type αγα polypeptide becomes incorporated into AP-2 complexes, which we denote AP-2 WT. Furthermore, the results presented here demonstrate that the mutant KKK/Q αγα polypeptide behaves indistinguishably from the wild-type, associating with the other AP-2 subunits and forming complexes (which we denote AP-2PPI) in the transiently transfected cells.

To investigate the cellular phenotype resulting from knockout of the PPI binding site of AP-2, we analyzed transfected BALB/c-3T3 cells by confocal fluorescence microscopy. Though the transfection efficiency of BALB/c-3T3 cells was lower than that of MOP-8 cells in our hands, the former were chosen for this experiment because their morphology after fixation is much more amenable to immunofluorescence analysis of plasma membrane coated pits. Cells transfected with wild-type (AP-2WT) or mutant KKK/Q (AP-2PPI) αγα constructs were double-labeled with mouse monoclonal antibody 100/3 to reveal the localization of the exogenous αγα product, and with either rabbit Ab31 or 27004 to localize endogenous α-adaptn or clathrin, respectively.

Fig. 6 a shows the localization of the AP-2WT αγα product at several different expression levels in transiently transfected cells. The vast majority of the expressed AP-2WT protein (upper panels) had a punctate distribution in the plane of the plasma membrane, with very little diffuse signal detectable. Comparison with the distribution of endogenous AP-2 α (lower panels) indicated almost complete colocalization (Fig. 6 a). The images also show that the presence of the γ hinge did not misdirect the protein to the Golgi region. Consistent with this finding, the AP-2WT distribution was also largely coincident with the localization of plasma membrane coated pits stained with anti-clathrin antibody, but did not colocalize with anti-clathrin staining in the trans-Golgi network (data not shown). Similar observations were made by Robinson (1993) on her initial use of the αγα construct for expression in Rat1 cells.

At low expression levels, AP-2WT had no detectable effect on the distribution of the endogenous α-adaptn (Fig. 6 a, left panels). Interestingly, in cells with higher levels of expression there is an apparent dominant-negative effect in that the level of endogenous α-adaptn in clathrin-coated pits is decreased compared with untransfected cells in the same field (Fig. 6 a, center and right panels). Only at

Figure 5. Wild-type and mutant KKK/Q AP-2 α polypeptides are associated with AP-2 subunits in intact cells. Equal amounts of lysates prepared under non-denaturing conditions from untransfected mouse MOP8 cells (mock) or cells transfected with either wild-type AP-2 αγα WT) or mutant KKK/Q constructs were double-labeled with mouse monoclonal antibody 100/3 to reveal the localization of the exogenous αγα product, and with either rabbit Ab31 or 27004 to localize endogenous α-adaptn or clathrin, respectively.

Figure 6. a Expressed AP-2 αWT, b Expressed AP-2 αPPI−

Endogenous AP-2 α

Expression levels of AP-2 WT, but not AP-2PPI−, is targeted normally to clathrin-coated pits at the plasma membrane. (a) AP-2WT expressed in BALB/c-3T3 cells exhibits a punctate pattern entirely coincident with that of endogenous P-2 α and is localized to plasma membrane clathrin-coated pits. With increasing levels of expression (right panels), the incorporation of the endogenous P-2 α polypeptide into coated pits is diminished. (b) Expressed AP-2PPI− does not colocalize with endogenous P-2 α in coated pits. With increasing expression of AP-2PPI−, correct localization of endogenous P-2 to plasma membrane clathrin-coated pits is also impaired (right panels). Bar, 10 μm.
unphysiologically elevated levels of expression is there any evidence for significant accumulation of soluble \( \text{AP-2}_{\text{WT}} \) protein (data not shown), and there is no detectable effect on the normal distribution of clathrin at the plasma membrane or in the Golgi region.

The localization of the mutant \( \text{AP-2}_{\text{PPI}} \) protein at several different levels of expression are shown in Figs. 6 b and 7. The distribution of the mutant protein differed radically from that of the wild-type protein. Generally, most of the \( \text{AP-2}_{\text{PPI}} \) localization was diffuse and at any level of expression, no significant amount of the mutant polypeptide could be detected in clathrin-coated pits at the plasma membrane. In some cells a small amount of finely punctate signal was detectable, most of which was intracellular. With few exceptions this signal did not coincide with that of endogenous \( \text{AP-2}_{\alpha} \) (Fig. 6 b), nor did it colocalize with either early or recycling endosomes (labeled with endocytosed fluorescent transferrin), or with the late endosome/lysosomal compartment (labeled with endocytosed fluorescent dextran) (data not shown). Interestingly, with increasing expression levels of the mutant \( \text{AP-2}_{\text{PPI}} \) protein, the proper localization of \( \text{AP-2} \) to discrete plasma membrane sites was diminished (Fig. 6 b, right panels).

Similarly, at low levels of mutant \( \text{AP-2}_{\text{PPI}} \) expression, the localization of clathrin to plasma membrane was not noticeably affected (Fig. 7, left and middle panels). However, clathrin localization was clearly abnormal at higher levels of mutant expression with a reduced number of plasma membrane coated pits present in comparison to adjacent, nonexpressing cells (Fig. 7, right panels). Interestingly, the clathrin signal in the Golgi region also seemed to be affected by elevated levels of \( \text{AP-2}_{\text{PPI}} \) expression, consistent with continuity between the plasma membrane and Golgi pools of clathrin.

Finally, we evaluated the internalization of the fluorescently tagged transferrin by cells expressing \( \alpha_{\gamma\delta\epsilon} \) constructs. In cells expressing low levels of either the \( \text{AP-2}_{\text{WT}} \) or \( \text{AP-2}_{\text{PPI}} \) mutant proteins, internalization of transferrin was indistinguishable from that in neighboring cells that were not expressing either product (data not shown), consistent with the absence of an effect on coated pit distribution in these cells. Interestingly, transferrin internalization was greatly diminished in cells expressing moderate levels of the mutant \( \text{AP-2}_{\text{PPI}} \) protein, consistent with the disruption of clathrin-coated pits in that population. In contrast, \( \text{AP-2}_{\text{WT}} \) did not detectably affect transferrin uptake until very high levels of expression were attained.

**Discussion**

In this study, we have sought to determine the importance of the high affinity PPI binding site located in the NH\(_3\)-terminal region of the \( \text{AP-2}_{\alpha} \) subunit in the process of receptor-mediated endocytosis. In previous reports we identified the polypeptide region involved in binding (Beck and Keen, 1991; Gaidarov et al., 1996) and provided in vitro evidence that PIP\(_3\), a product of phosphatidylinositol 3-kinase, is the ligand of highest known affinity for \( \text{AP-2} \) in assembled coat structures (Gaidarov et al., 1996). Though these results suggested that PIP\(_3\) is a physiologically relevant ligand for \( \text{AP-2} \), in the absence of definitive data from intact cells this conjecture remains uncertain, as does the precise identity of other ligand(s) that may interact with \( \text{AP-2} \). Accordingly, we undertook a complementary approach to study the physiological function of the \( \text{PPI} \) binding site. We sought to identify and alter amino acid residues critical for PPI binding to \( \text{AP-2} \), and to evaluate the effects of expression of this mutant \( \text{AP-2} \) in intact cells. The results of these efforts lead to the conclusion that an active PPI site is indeed required for \( \text{AP-2} \) function in receptor-mediated endocytosis.

In some PPI-binding proteins short peptides (8–20 residues) have been found to be sufficient for high affinity binding of inositol phosphates or phosphoinositides. Examples include certain actin-associated proteins such as gelsolin (Janmey et al., 1992; Yu et al., 1992) and profilin (Raghunathan et al., 1992; Sohn et al., 1995) and some C2 domain-containing proteins such as synaptotagmin (Fukuda et al., 1994, 1995). Pleckstrin homology (PH) domains, which are found in a number of proteins involved in signal transduction and are believed to function in membrane recruitment and regulation of enzymatic activity (Shaw, 1996; Fukuda and Mikoshiba, 1997; Lemmon and Ferguson, 1998), provide a contrasting pattern. In these proteins essentially the entire ~100 amino acid module is necessary for high affinity ligand binding.

The mutagenesis analysis reported here suggests that the latter characterization is more applicable to the PPI binding site in \( \text{AP-2}_{\alpha} \). It has a highly organized secondary structure and seems to require a \( \approx 60 \) residue region for full binding activity, from which we infer that this portion of the \( \alpha \) structure comprises a distinct structural and functional domain. Positively charged amino acids throughout the region contribute to the binding interaction (Fig. 2), with two clusters of basic residues toward each end (a lysine triad at 55–57 and K31/R32/K35) appearing to be most important. In parallel with the PH domains whose tertiary structure in complex with ligands has been determined (e.g., that in \( \beta \)-spectrin; Hyvonen et al., 1995), the PPI binding region in \( \text{AP-2} \) may be a large positively charged surface with some residues in direct contact with the bound ligand, while others may be responsible for the initial electrostatic recruitment of the PPI to the binding pocket or the formation of the charged surface. It is in-
creasingly appreciated that protein domains may have remarkably similar three-dimensional structure but share very limited sequence homology. Consequently, the relationship of the A-P2 binding site to other PPI binding domains with which it does not share detectable sequence identity will probably only be answered after determination of its tertiary structure.

This region of the A-P2 α sequence, and the basic residues in particular, are virtually conserved in both Drosophila and C. elegans homologues of mammalian αA. Furthermore, although the overall identity of two recently identified yeast α homologues with the mammalian protein in this region is 30–40%, most of the basic residues required for inositol binding in the mammalian protein, in particular the lysine triad, are also conserved (Fig. 8). This extends the inference of a functional PPI binding domain to these lower eukaryotes. Interestingly, the mammalian A-P1 γ, A-P3 δ, and recently identified ε subunit of a novel A-P4 complex show distinct but considerably less conservation of several of these basic residues (Fig. 8) to the best of our knowledge the PPI binding properties of these proteins have not been reported. Finally, the COP1 coatamer (Chaudhary et al., 1998) and A-P180 (Y e et al., 1995) also bind PPIs but have no discernible sequence similarity to A-P2 α. Collectively, these observations suggest that PPI binding by coat subunits involved in membrane transport is a ubiquitous phenomenon, and that the nature of specific residues in this binding domain may impart inositol binding specificity.

There is increasing evidence for an essential role of phosphoinositides in transport vesicle function at different locations in mammalian cells. Phosphoinositides, particularly PIP2, formed secondarily to ARF activation of phospholipase D, have been implicated in the recruitment of COP1 coat proteins onto the membranes of the Golgi stacks (Donaldson et al., 1992; Palmer et al., 1993; Ktis-takis et al., 1996). The specific interaction between these acidic phospholipids and coatamer, which has been shown to bind PPIs and particularly PIP3 with high affinity (Fleischer et al., 1994; Chaudhary et al., 1998), could contribute to recruitment of the coatomer to a specific membrane location, though this is controversial (Stamnes et al., 1998). With regard to A-P2, broken cell assays have shown that PIP2 sequestration, accomplished either pharmacologically with neomycin or biochemically using the PH domain of PLC, had an inhibitory effect on A-P2 recruitment to the plasma membrane, indirectly implicating phosphoinositides in A-P2 targeting (West et al., 1997; Jost et al., 1998).

Our in vitro binding data indicated that the (assembled) coat form of A-P2 shows the highest affinity for phosphoinositides, as compared with inositol phosphates, and that the converse is true for the soluble (disassembled) A-P2 protein (Beck and Keen, 1991; Gaidarov et al., 1996). These observations suggest that the presence of phosphoinositides will drive the A-P2 molecule toward its higher affinity, assembled form. This conjecture is supported by the observation reported here that A-P2 lacking a functional PPI site is not incorporated into coated pits. Further, it has been reported that the receptor cytoplasmic tail interaction of A-P2 with bound phosphoinositide is comparable to that with A-P2 in an assembled coat structure, and that both are of higher affinity than that with free A-P2 (Rapport et al., 1998), again suggesting that inositol binding drives A-P2 toward an assembled conformation thereby promoting its ability to interact with clathrin.

The results reported here provide direct support for the notion that PPIs play a physiologically important role in membrane recruitment of A-P2. The mutant A-P2ppi⁻ assayed in vitro is almost totally defective in PPI binding, but otherwise indistinguishable from the wild-type protein by multiple structural and functional criteria. However, in intact cells A-P2ppi⁻ is almost completely defective in incorporation into plasma membrane clathrin-coated pits. Unlike the wild-type protein, it tends to have a diffuse distribution throughout the cell (Figs. 6 a and 7). At high expression levels, a small amount of punctate signal is also detectable which may reflect the inability of A-P2ppi⁻ to bind PPIs and resist self-association (Beck and Keen, 1991). These observations are generally consistent with earlier results of Page and Robinson (Page and Robinson, 1995). Using α/γ chimeras, their results indicated that the plasma membrane/Golgi targeting signals are localized primarily between residues 130 and 330–350 in the α and γ sequences, respectively. Interestingly, chimeric proteins in which 132, or even 36, residues from the NH2-terminal region of the A-P2α subunit had been replaced by corresponding γ sequences gave substantial diffuse signal and considerably reduced, though still detectable, recruitment to plasma membrane coated pits (for example see Page and Robinson, 1995; Fig. 3, A and B). This may reflect cooperation of the plasma membrane targeting signal, localized by these workers to the distal α sequence, with the action of a hypothetical PPI binding domain in the NH2-termi

![Figure 8. Basic residues involved in PPI binding in mammalian A-P2 α are highly conserved.](image-url)
terminal region of the γ sequence (see above and Fig. 8). A coiled-coil to this reasoning, the A P-2PPI is not detectably recruited to coated pits despite presence of a plasma membrane targeting signal because it lacks PPI binding.

Interestingly, A P-2PPI also acts as a dominant-negative inhibitor of coated pit formation. This suggests that excess inactive A P-2PPI complexes effectively sequester the other A P-2 subunits and/or occupy the limited sites that must be available for coat formation (M oore et al., 1987; Santini et al., 1998; Gaidarov et al., 1999; Subtil et al., 1999), indicating in either case that the binding to A P-2 of PIP₃ or another specific PPI ligand in the membrane is important early during the receptor mediated endocytosis process, i.e., at the stage of clathrin-coated pit formation.

We have recently demonstrated that clathrin-coated pits form at specific and defined sites on the plasma membrane, and that a cytoskeletal framework in tight association with the membrane likely plays a major organizational role in this process (Gaidarov et al., 1999). Together, these results suggest a model in which coat formation is initiated and anchored by interactions oriented both outward toward the plasma membrane and inward toward a neighboring skeletal structure.

This general model is supported by our recent demonstration that PPIs are also involved in the ligand-dependent internalization of another class of receptors, the G-protein-coupled receptors. Nonvisual arrests, which have been shown to act as adaptors in the internalization of β₂-adrenergic receptors (Ferguson et al., 1996; Goodman et al., 1996), bind PPIs with high affinity. We found that soluble PPIs and phosphoinositides differentially modulate arrestin interaction with clathrin and receptor. Furthermore, as in the case of the A P-2 adaptor reported here, a functional PPI binding site is critical to the ligand-dependent recruitment of the receptor-arrestin complex to clathrin-coated pits (Gaidarov et al., 1999).

Together, these findings point to common themes of phosphoinositol action in membrane trafficking events: they may serve either as recruitment signals for coat components and/or to modulate the interaction of coat components with receptor complexes. The presence in clathrin-coated pits and vesicles of synaptotagmin I (Haffne et al., 1997), a phosphoinositide 5-phosphatase, suggests that adaptor functions may be regulated by a complex interplay of different enzymes involved in site-specific phosphoinositide metabolism. A ditional enzymes involved in adaptor/coat regulation, and the factors, which modulate their activity, are yet to be discovered.

We are grateful to Dr. E. DeSteban Dell’Anglica (NIHR) for sharing unpublished information, to Drs. Oscar Goodman for help with cage binding experiments, Dr. J. Johansson (University Pennsylvania) for help with CD spectroscopy, Dr. M. Robinson (Cambridge, UK) for cDNA constructs and antibodies to A P-2 subunits, and to Dr. A. Sorkin (University of Colorado) for antibodies against α-adaptin.

Supported by National Institutes of Health grant GM-49217 to J. H. Keen.

References


