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Phosphoinositide–AP-2 Interactions Required for Targeting to Plasma Membrane Clathrin-coated Pits

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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 NH$_2$-terminal region of the AP-2 $\alpha$ subunit (Gaidarov et al. 1996. J. Biol. Chem. 271:20922-20929). Here we used deletion and site-directed mutagenesis to determine that $\alpha$ 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 $\alpha$ 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 nonglycosylated protein component of the coat is the multimeric protein complex termed adaptor or AP$^1$, 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 ($\gamma$ and $\beta'$ in AP-1 and $\alpha$ and $\beta$ in AP-2), a medium subunit ($\mu_1$ in AP-1 and $\mu_2$ in AP-2), and a small subunit ($\sigma_1$ in AP-1 and $\sigma_2$ in AP-2). The $\alpha$ subunits have some homology to the $\gamma$ subunit and both are very distantly related to the $\beta/\beta'$ 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. AP-2 interacts with clathrin through the $\alpha$ and $\beta$ subunits (Goodman and Keen, 1995; Shih et al., 1995) and promotes coat formation (Prasad and Keen, 1991). Interaction of the AP-2 $\mu$ 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; R odionov 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., 1998), amphiphysin (David et al., 1996), and eps15 (Benmerah et al., 1996; Iannolo et al., 1996) are critical.
Materials and Methods

Materials

Clathrin was purified from bovine brain-coated vesicles as described (Keen, 1987). o-myo-Inositol hexakisphosphate (IP₆) was obtained from Calbiochem. [³H]IP₆ was from DuPont NEN. ³⁵S-Translabel was from ICN, l-3-tyrosylamido-2-phenylethyl chloromethyl ketone-trypsin was from Worthington Biochemical, Inc. Restriction and modification enzymes 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 α5-80

Deletion and site-directed mutations in the α5-80 insert in the plasmid pMal-c2 (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 α5-80 Peptides

Purified maltose-binding protein chimera 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).

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was performed on an Aviv-62DS spectrophotometer 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 Anrude et al. (1993).

In Vitro Transcription-Translation of Wild-type and Mutant AP-2α₅₄

Wild-type and mutant α₅₄ subunits were expressed in vitro using a TnT rabbit reticulocyte lysate transcription-translation system. First, plasmid pSP65α₅₄ (Goodman and Keen, 1995) was modified to optimize expression by removing most of the 5′ untranslated region beyond 10 bp upstream of the initiation codon of α₅₄ cDNA. A nuclease digest showed that the resulting plasmid pSP65α₄ gave 5–10-fold higher expression than pSP65α₅₄. M utant α₅₄ cDNA fragments were cloned into the pSP65α₄ vector by a series of subcloning procedures, the details of which are available on request. The resulting plasmid pSP65α₅₄K KK K-O and the plasmid pSP65α₄ were used for in vitro expression of mutant and wild-type α₅₄.

Transcription-translation reactions were performed according to the manufacturer’s recommendations in the presence of ³⁵S-Translabel. A 250 ml reaction mixture contained 100,000 rpm for 20 min at 4°C in a TLA 100 rotor (Beckman).

Proteolysis and Clathrin Cage Binding Assays of In Vitro Translated α Polypeptides

Limited tryptic proteolysis and clathrin cage binding experiments with in vitro–translated wild-type and mutant AP-2α₅₄ polypeptides were performed essentially as described previously (Goodman and Keen, 1995).

Expression of Wild-type and Mutant Adaptin Constructs in Mammalian Cells

Plasmid containing the α₅₄ construct (derived from bovine AP-2α₅₄) in
Confocal microscopy was performed on a Bio-Rad MRC-1024 laser scanning confocal microscope, using a Zeiss Plan-Apo 63× oil immersion objective. Images were collected sequentially in the photon counting mode using single line excitation.

**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 A P-2 to the region between residues 5 and 80 at the NH$_2$-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 IP$_6$ 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. A s 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 IP$_6$ binding. A cordingly, we produced a series of fusion proteins of MBP with the A P-2 α5-80 fragment (de noted MBP-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. E ach of these fusion proteins was purified by affinity chromatography and tested for IP$_6$ binding.

We found that residues scattered throughout the α 5–80 region affected IP$_6$ binding, though to differing extents (Fig. 2). The mutations could be divided into several groups in terms of their effects on IP$_6$ binding: no reduc-

| MBP | IP$_6$ Binding | K$_D$ (nM) |
|-----|---------------|------------|
| 5   | + +           | 130        |
| 5   | -             | -          |
| 21  | -             | -          |
| 5   | + +           | 150        |

Figure 1. A PPI binding site in the NH$_2$-terminal region of A P-2 α. I ndicated segments of the A P-2α sequence coupled at their NH$_2$ terminus to MBP were analyzed for $[^3]$H]IP$_6$ binding and approximate binding affinity. A high affinity site is present within residues 21–80, while shorter segments do not retain specific binding activity.

**Immunofluorescence Microscopy Analysis**

Immunofluorescence analysis was performed as described previously (Santini and K een, 1996). In brief, cells were washed, fixed, permeabilized, and exposed to mouse monoclonal antibody 100/3 (50 μg/ml) for detection of α$_{γ γ}$ polypeptide and rabbit A b31 (1:150) for detection of endogenous A P-2 (Sorkin et al., 1995). We have found that residues scattered throughout the α 5–80 region affected IP$_6$ binding, though to differing extents (Fig. 2). The mutations could be divided into several groups in terms of their effects on IP$_6$ binding: no reduc-

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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. 3 a. 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. Also shown in Fig. 3 a 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 (Sonnichsen et al., 1992). Under these conditions, the spectrum of the wild-type fragment exhibited much more pronounced maximum and minima, corresponding to almost 100% α-helicity.

The CD spectrum of the mutant A P-2 α5-80-KKK/Q fragment is presented in Fig. 3 b, along with the spectrum of the wild-type peptide. The mutant α5-80-KKK/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-K56E 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
amino acid substitutions, but rather results from disruption of direct interactions between the ligand and basic residues on the protein.

We tried to determine whether ligand binding induces any conformational change in the PPI binding domain α5-80. Unfortunately, in the presence of IP₃ at concentrations as low as 1 μM the peptide aggregated. This problem could not be overcome by the addition of salt and/or non-ionic detergents.

**Structural and Functional Characterization of the KKK/Q Mutant in the Context of the Full-length In Vitro Translated AP-2 α Subunit**

To further evaluate the effects of the KKK/Q mutation on the overall properties of the AP-2α subunit, we performed structural and functional assays on full-length wild-type and mutant AP-2α polypeptides generated in a rabbit reticulocyte in vitro translation system. We have previously shown that the in vitro translated AP-2α polypeptide is folded similarly to that in the native AP-2 complex isolated from bovine brain (Goodman and Keen, 1995). For example, limited tryptic proteolysis of in vitro-translated AP-2α generates fragments of 55-66 kD and 40 kD, corresponding to the NH₂-terminal core and COOH-terminal appendage domain generated on similar treatment of the native bovine brain AP-2 (Zaremba and Keen, 1985). Similar limited proteolysis of in vitro-translated mutant KKK/Q α₀ polypeptide produced a pattern with the characteristic core and 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 NH₂-terminal region of the AP-2α polypeptide does not cause gross misfolding of the entire subunit on synthesis.

The isolated AP-2α 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 AP-2α polypeptide required for this interaction. We found that binding of the in vitro-translated mutant KK(K/Q) α₀ polypeptide to clathrin cages was indistinguishable from that of the wild-type polypeptide (Fig. 4b). Collectively, these results indicate that the mutant KK(K/Q) AP-2α polypeptide retained the native tertiary structure and function of the wild-type protein, but that it is essentially devoid of PPI binding ability.

**Functional Characterization of Mutant KKK/Q AP-2α in Intact Cells**

To investigate the functional role of the AP-2α PPI binding site in intact cells, we employed an αγκ construct described previously (Robinson, 1993) and kindly provided by Dr. M. S. Robinson (University of Cambridge). The αγκ construct encodes mouse α₀-polypeptide in which the hinge region between the core and appendage domains, corresponding to α₀ residues 620-700, has been substituted with the hinge region of the bovine Golgi-specific AP-1γ subunit. This enabled us to specifically localize the expressed αγκ polypeptides in transiently transfected mouse fibroblasts using a γ-specific monoclonal antibody (mAb 100/3) which does not recognize the endogenous (mouse) protein (Ahle et al., 1988). Additionally, the endogenous AP-2α polypeptide could also be uniquely localized using A31, a rabbit polyclonal anti-α antibody kindly provided by Dr. A. Sorkin (University of Colorado). We found that though A31 was produced by inoculation with a fragment consisting of the hinge and appendage domains of the rat brain α₀ subunit (Sorkin et al., 1995), it reacts only with the α hinge region and not with the α appendage (see Materials and Methods). Thus, the endogenous and the transiently expressed exogenous α polypeptides could be detected independently, providing important tools for the study of mutant α subunits.

First, we asked whether wild-type and KKK/Q mutant AP-2α polypeptides expressed after transfection are incorporated into AP-2 adaptor complexes in intact cells. Lysates of mock, wild-type αγκ, and KKK/Q mutant αγκ-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 AP-2 subunits. As shown in Fig. 5, reactivity with the anti-γ 100/3 antibody was detected only in immunoprecipitates from cells transfected with the wild-type or mutant αγκ constructs, consistent with the inability of this antibody to recognize the endogenous mouse AP-2γ 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/ Δαγα 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-2WT. Furthermore, the results presented here demonstrate that the mutant KKK/Q/ Δαγα 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-adaptin 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 Δαγα construct for expression in Rat1 cells.

At low expression levels, AP-2WT had no detectable effect on the distribution of the endogenous α-adaptin (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-adaptin in clathrin-coated pits is decreased compared with untransfected cells in the same field (Fig. 6 a, center and right panels). Only at
unphysiologically elevated levels of expression is there any evidence for significant accumulation of soluble A P-2WT 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 A P-2ppi− 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 A P-2ppi− 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 A P-2 α (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 A P-2ppi− protein, the proper localization of A P-2 to discrete plasma membrane sites was diminished (Fig. 6 b, right panels).

Similarly, at low levels of mutant A P-2ppi− 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 A P-2ppi− 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 αγγα constructs. In cells expressing low levels of either the A P-2WT or A P-2ppi− 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 A P-2ppi− protein, consistent with the disruption of clathrin-coated pits in that population. In contrast, A P-2WT 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 NH2-terminal region of the A P-2α 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 P1-3,4,5-P3, a product of phosphatidylinositol 3-kinase, is the ligand of highest affinity for A P-2 in assembled coat structures (Gaidarov et al., 1996). Though these results suggested that PIP3 is a physiologically relevant ligand for A P-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 A P-2. A accordingly, we undertook a complementary approach to study the physiological function of the A P-2 PPI binding site. We sought to identify and alter amino acid residues critical for PPI binding to A P-2, and to evaluate the effects of expression of this mutant A P-2 in intact cells. The results of these efforts lead to the conclusion that an active PPI site is indeed required for A P-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; Y u 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 A P-2 α. It has a highly organized secondary structure and seems to require a 60-residue region for full binding activity, from which we infer that this portion of the α 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 β-spectrin; Hyvonen et al., 1995), the PPI binding region in A P-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 P-2 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 P-2 α sequence, and the basic residues in particular, are virtually conserved in both D.rosophila 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 P-1 γ, A P-3 δ, and recently identified ε subunit of a novel A P-4 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 COPI coatamer (Chaudhary et al., 1998) and A P180 (Y e et al., 1995) also bind PPIs but have no discernible sequence similarity to A P-2 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 A RF activation of phospholipase D, have been implicated in the recruitment of COPI coat proteins onto the membranes of the Golgi stacks (Donaldson et al., 1992; Palmer et al., 1993; K tis 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 (F lie scher et al., 1994; Chaudhary et al., 1998), could contribute to recruitment of the coatamer to a specific membrane location, though this is controversial (St amness et al., 1998).

With regard to A P-2, broken cell assays have shown that PIP2 sequestration, accomplished either pharmacologically with neomycin or biochemically using the PH domain of PLCζ, had an inhibitory effect on A P-2 recruitment to the plasma membrane, indirectly implicating phosphoinositides in A P-2 targeting (West et al., 1997; J ost et al., 1998).

Our in vitro binding data indicated that the (assembled) coat form of A P-2 shows the highest affinity for phosphoinositides, as compared with inositol phosphates, and that the converse is true for the soluble (disassembled) A P-2 protein (Beck and K een, 1991; G aidarov et al., 1996). These observations suggest that the presence of phosphoinositides will drive the A P-2 molecule toward its higher affinity, assembled form. This conjecture is supported by the observation reported here that A P-2 lacking a functional PPI site is not incorporated into coated pits. Further, it has been reported that the receptor cytoplasmic tail interaction of A P-2 with bound phosphoinositide is comparable to that with A P-2 in an assembled coat structure, and that both are of higher affinity than that with free A P-2 (R apport et al., 1998), again suggesting that inositol binding drives A P-2 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 P-2. The mutant A P-2PP which 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 P-2PP 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. 6a and 7). A t high expression levels, a small amount of punctate signal is also detectable which may reflect the inability of A P-2PP to bind PPIs and resist self-association (Beck and K een, 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-terminus of A P-2α 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-terminus of A P-2α.

Figure 8. Basic residues involved in PPI binding in mammalian A P-2 α are highly conserved. Sequence alignments of mouse A P-2 α (M ouse α, accession number P17426) with Drosophila melanogaster (D m α, accession number Y 13092), Caenorhabditis elegans (C e α, accession number U 28742), Saccharomyces cerevisiae (S c α, accession number P38065), Schizosaccharomyces pombe (S p α, accession number A B004535), mouse γ adaptin (mouse γ, accession number X 54424), human δ (accession number A F002163), and human ε (I. M.A.G.E. Clone ID 1031294; D e/l’A ngelica, E., personal communication). Sequence comparisons were performed with PILEUP, with numbering according to the mouse A P-2 α sequence. Residues identical to the mouse A P-2 α protein in four or more other sequences are highlighted on a black background; nonidentical but conserved residues in three or more sequences are shaded. Asterisks denote basic residues critical to PPI binding in the A P-2 α sequence.
ter terminal region of the γ sequence (see above and Fig. 8). A
according to this reasoning, the AP-2PPI is not detectably
recruited to coated pits despite presence of a plasma mem-
brane targeting signal because it lacks PPI binding.

Interestingly, a AP-2PPI also acts as a dominant-negative
inhibitor of coated pit formation. This suggests that
excess inactive AP-2PPI complexes effectively sequester
the other AP-2 subunits and/or occupy the limited sites
that must be available for coat formation (Moor et al.,
1987; Santini et al., 1998; Gaidarov et al., 1999; Subtil et al.,
1999), indicating in either case that the binding to AP-2 of
PPI or another specific PPI ligand in the membrane is im-
portant 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 mem-
brane, and that a cytoskeletal framework in tight asso-
ciation with the membrane likely plays a major organi-
zational role in this process (Gaidarov et al., 1999).
Together, these results suggest a model in which coat for-
mation is initiated and anchored by interactions oriented
both outward toward the plasma membrane and inward
and toward a neighboring skeletal structure.

This general model is supported by our recent demon-
stration that PPIs are also involved in the ligand-depen-
dent internalization of another class of receptors, the
G-protein-coupled receptors. Nonvisual arrestins, which
have been shown to act as adaptors in the internalization of
β2-adrenergic receptors (Ferguson et al., 1996; Good-
man 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
phosphoinositide action in membrane trafficking events:
they may serve either as recruitment signals for coat com-
ponents and/or to modulate the interaction of coat compo-
nents with receptor complexes. The presence in clathrin-
coated pits and vesicles of synaptotagmin (Haffner et al.,
1997), a phosphoinositide 5-phosphatase, suggests that
adaptor functions may be regulated by a complex inter-
play of different enzymes involved in site-specific phos-
phoinositide metabolism. A dDitional enzymes involved in
adaptor/coat regulation, and the factors, which modulate
their activity, are yet to be discovered.

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References

Andrade, M. A., P. Chacon, J. J., Merelo, and F. Moran. 1993. Evaluation of sec-
ondary structure of proteins from UV circular dichroism spectra using an
unsupervised learning neural network. Protein Eng. 6:383–390.

Barlow, A. B., D. Binns, K. L. Lin, A. M. A. Tinson, D. M. Jameson, H. L. Yin, and J. P. Albane. 1998. Synergistic activation of dynamin GTPase by Grb2 and
phosphoinositides. J. Biol. Chem. 273:3791–3797.

Beck, K. A., and J. H. Keen. 1991. Interaction of phosphoinositide cycle inter-
mediates with the plasma membrane-associated clathrin assembly protein
AP-2. J. Biol. Chem. 266:4442–4447.

Benmenah, A., B. Begue, A. Daubert-Varsat, and N. Cerf-Bensussan. 1996. The
ear alpha of adaptin interacts with the COOH-terminal domain of the Eps
15 protein. J. Biol. Chem. 271:8121–8126.

Boll, W., H. Ōno, Z. Songyang, I. Rapport, L. C. Cantley, J. S. Bonifacino,
and T. Kirchhausen. 1996. Sequence requirements for the recognition of ty-
rosine-based endocytic signals by clathrin-2 complexes. EMBO J (Eu-
ropean Molecular Biology Organisation) 15:7589–7601.

Brown, W. J., D.B. DeWald, S.D. Emr, H. Plutner, and W. E. Balch. 1995. Role
for phosphatidylinositol 3-kinase in the sorting and transport of newly
synthesized lysosomal enzymes in mammalian cells. J. Cell Biol. 130:781–796.

Chaudhury, A., Q. M. Gu, O. T. Hay, A. A. Profit, Y. Qi, L. J. Jayakumar, S. Flei-
scher, and G. D. Prestwich. 1998. Specific interaction of Golgi coatomer pro-
tein alpha-Cop with phosphatidylinositol 3,4,5-trisphosphate. J. Biol.
Chem. 273:6344–6350.

David, C., P. S. McPherson, O. Mundling, and P. de Camilli. 1996. A role of am-
phiphysin in synaptic vesicle endocytosis suggested by its binding to dy-
namin in nerve terminals. Proc. Natl. Acad. Sci. USA. 93:331–335.

Davidson, H. W. 1995. Wortmannin causes mCystaration of phosphoinos 
. D. evi-
dence for the involvement of a phosphatidylinositol 3-kinase in vesicular
transport to lysosomes. J. Cell Biol. 130:797–805.

Donaldson, J. G., D. Cassel, R. A. Kahn, and R. D. Klausner. 1992. A DP-ribo-
sylation factor, a small GTP-binding protein, is required for binding of the
coatomer protein beta-COP to Golgi membranes. Proc. Natl. Acad. Sci.
USA. 89:6408–6412.

Ferguson, S.S., W.W. Dowe, Jr., A. G. Colapietro, L. S. Barak, L. Menard, and M. G. Caron. 1996. Role of beta-arrestin in mediating agonist-promoted G
protein-coupled receptor internalization. Science. 271:363–366.

Fleischer, B. J., X. M. Yarleffin, S.B. Shears, D. J. Palmer, and A. Fleischer.
1994. Golgi coatomer binding, and forms K{\textopenbrace} selective channels gated by,
inositol polyphosphates. J. Biol. Chem. 269:17826–17832.

Fukuda, M., J. A ruga, M. Niinobe, S. A imoto, and K. Mikoshiba. 1994. Inositol
1,3,4,5-tetrakisphosphate binding to C2 domain of IP4BP/synaptotagmin
II. J. Biol. Chem. 269:29206–29211.

Fukuda, M., T. Kojima, J. A ruga, M. Niinobe, and K. Mikoshiba. 1995. Func-
tional diversity of C2 domains of synaptotagmin family. Mutational anal-
ysis of inositol high polyphosphate binding domain. J. Biol. Chem. 270:26523–
26527.

Fukuda, M., and K. Mikoshiba. 1997. The function of inositol high poly-
phosphate binding proteins. Bioessays. 19:593–603.

Gaidarov, I., Q. Chen, J. R. Falck, K. K. Reddy, and J. H. Keen. 1996. A func-
tional phosphatidylinositol 3,4,5-trisphosphate-binding domain in the clathrin
adaptin A P-2 alpha subunit. Implications for the endocytic pathway (pub-
lished erratum appears in J. Biol. Chem. 271:2188). J. Biol.
Chem. 271:20922–20929.

Gaidarov, I., J. G. Kruptin, J. R. Falck, J. L. Benovic, and J. H. Keen. 1999. A
reabin function in G protein-coupled receptor endocytosis requires phos-
phoinositide binding. EMBO J (European Molecular Biology Organisation) 18:1891–1901.

Gaidarov, I., F. Santini, R. A. Warren, and J. H. Keen. 1999. Spatial control of
coated-pit dynamics in living cells. Nat. Cell Biol. 1:1–7.

Goldman, L. A., R. Rottapal, and J. S. Bergner. 1997. Phosphatidylinositol 3-
kinase and C2a{\textopenbrace} influx dependence for ligand-stimulated internalization of
the C-k receptor. J. Biol. Chem. 272:30519–30525.

Goodman, O. B., Jr., and J. H. Keen. 1995. The alpha chain of the AP-2 adaptor
is a clathrin binding subunit. J. Biol. Chem. 270:23768–23773.

Hafler, C. K., T. Ataki, H. Chen, N. Ringstad, A. Hudson, M. H. Butler, A. E.
Salcini, P. P. Di Fiore, and P. de Camilli. 1997. Synaptotagmin 1: localization on
covaled coated endocytic intermediates in nerve terminals and interaction of its
170 kDa isoform with Eps15. J. Biol. Chem. 272:30519–30525.

Haffner, C. K., T. Ataki, H. Chen, N. Ringstad, A. Hudson, M. H. Butler, A. E.
Salcini, P. P. Di Fiore, and P. de Camilli. 1997. Synaptotagmin 1: localization on
covaled coated endocytic intermediates in nerve terminals and interaction of its
170 kDa isoform with Eps15. J. Biol. Chem. 272:30519–30525.
Schu, P.V., K. Takegawa, M.J. Fry, J.H. Stack, M.D. Waterfield, and S.D. Emr. 1995. Phosphatidylinositol 3-kinase activity is required for a postendocytic step in protein-derived growth factor receptor trafficking. J. Biol. Chem. 270:13225–13230.

Joly, M., A. Kazlauskas, and S. Corvera. 1995. Phosphatidylinositol 3-kinase activity is required for the formation of constitutive transport vesicles from the Tgn. J. Cell Biol. 139:339–349.

Jones, S.M., and K.E. Howell. 1997. Phosphatidylinositol 3-kinase is required for endocytosis of coated vesicle formation. Curr. Biol. 7:1399–1402.

Kee, J.H. 1987. Clathrin assembly proteins: affinity purification and a model for coat assembly. J. Cell Biol. 105:1899–1998.

Kee, J.H. 1990. Clathrin and associated assembly and disassembly proteins. Annu. Rev. Biochem. 59:415–438.

Kotakas, N.T., H.A. Brown, M.G. Waters, P.C. Sternweis, and M.G. Roth. 1996. Evidence that phospholipase D mediates an ADP ribosylation factor-dependent formation of Golgi coated vesicles. J. Cell Biol. 134:295–306.

Lemmon, M.A., and K.F. Ferguson. 1998. Pleckstrin homology domains. Topics in Microbiol. Immunol. 228:39–74.

Lemmon, M.A., and J.H. Keen. 1998. Clathrin-coated vesicle formation and protein sorting: an integrated process. J. Biol. Chem. 273:964–967.

Lemmon, M.A., and K.M. Ferguson. 1998. Pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. Annu. Rev. Biochem. 67:169–212.

Lemmon, M.A., and J.H. Keen. 1999. The pleckstrin homology domain of dynamin: implications for stimulation of GTPase activity. Proc. Natl. Acad. Sci. USA. 96:10079–10083.

Lemmon, M.A., and J.H. Keen. 1995. Syntaptotagmin I is a high affinity receptor for clathrin AP-2: implications for the pH domain of dynamin. Proc. Natl. Acad. Sci. USA. 92:13327–13332.

Shaw, G. 1996. The pleckstrin homology domain: an intriguing multifunctional protein module. Bioessays. 18:35–46.

Shih, W.-A., A. Gullasser, and T. Kirchhausen. 1995. A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes. J. Biol. Chem. 270:31083–31090.

Shiratori, T.S., M. Iyatake, H. Ohno, C. Nakaseko, K. Isono, J.S. Bonifacino, and T. Saito. 1997. Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. Immunity. 6:583–589.

Schiavo, G., Q.M. Gu, G.D. Prestwich, T.H. Sollner, and J.E. Rothman. 1996. Localization of a binding site for phosphatidylinositol 4,5-bisphosphate on human profilin. J. Biol. Chem. 270:21114–21120.

Sorrentino, F. Santini, and J.H. Keen. 1996. Endocytosis of activated receptors and clathrin assembly protein AP-2 are related proteins that form posttranslational selectivity ion channels in planar lipid bilayers. Proc. Natl. Acad. Sci. USA. 89:8976–8980.

Sorkin, A., T. M. Kinsky, W. Shih, T. Kirchhausen, and G. Carpenter. 1995. Stoichiometric interaction of the epidermal growth factor receptor with the clathrin-associated protein complex AP-2. J. Biol. Chem. 270:619–625.

Stack, J.H., and S.D. Emr. 1994. Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific 3′-kinase activities. J. Biol. Chem. 269:31552–31562.

Stack, J.H., P.K. Herman, P.V. Shu, and S.D. Emr. 1993. A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. EMBO J. (Eur. Mol. Biol. Organ.) 12:2195–2204.

Stack, J.H., T.C. Sudhof, and R.G. Anderson. 1994. The pleckstrin homology domain of dynamin is an inositol polyphosphate binding protein: isolation and characterization. FEBS Lett. 359:93–98.

Stack, J.H., and S.D. Emr. 1993. Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science. 260:89–91.

Shaw, G. 1996. The pleckstrin homology domain: an intriguing multifunctional protein module. Bioessays. 18:35–46.

Shih, W.-A., A. Gullasser, and T. Kirchhausen. 1995. A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes. J. Biol. Chem. 270:31083–31090.

Shiratori, T., S. Iyatake, H. Ohno, C. Nakaseko, K. Isono, J.S. Bonifacino, and T. Saito. 1997. Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. Immunity. 6:583–589.

Sohr, R.H., J. Chen, K.S. Kobilan, P.F. Bray, and P.J. Goldschmidt-Clermont. 1995. Localization of a binding site for phosphatidylinositol 4,5-bisphosphate on human profilin. J. Biol. Chem. 270:21114–21120.

Sonnichsen, F.D., J.E. Van Eyk, R.S. Hodges, and B.D. Sykes. 1992. Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. Biochemistry. 31:8790–8796.

Sorkin, A., T. M. Kinsky, W. Shih, T. Kirchhausen, and G. Carpenter. 1995. Stoichiometric interaction of the epidermal growth factor receptor with the clathrin-associated protein complex AP-2. J. Biol. Chem. 270:619–625.

Stack, J.H., and S.D. Emr. 1994. Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities. J. Biol. Chem. 269:31552–31562.